University of Alberta

Immune Status in Male Cyclists during Heavy Endurance Training

By

Shona Elise Moss Lovshin ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of Master of Science.

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Shona Moss Lovshin

Permanent address:

27 Ossington Avenue
Ottawa, Ontario
K1S 3B3

22 - 8 - 97

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Immune Status in Male Cyclists during Heavy Endurance Training" submitted by Shona Elise Moss Lovshin in partial fulfillment of the requirements for the degree of Master of Science

Supervisor: S.R. Petersen

Gordon J Bell
G.J. Bell

Bhambhani
Y. Bhambhani

J.L. Hannon

Date: August 5, 1997

Dedication

To my husband Stephen Lovshin and to my parents John and Shirley Moss.

Abstract

The hypothesis that heavy endurance training suppresses the immune system and alters resting concentrations of urinary cortisol and serum testosterone was tested in nine experienced male cyclists. Absolute counts for lymphocyte subsets ($CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^-CD16^+CD56^+$, and $CD19^+$), cytokine concentrations (IL-1β, IL-6, and TNFα), and serum testosterone concentrations were determined on post-exercise (36-44 h) morning blood samples. Cortisol concentrations were determined on post-exercise (24-26 h) 24 h urine collections. Samples were collected before and after four consecutive training phases of the study: 4 wk baseline, 6 wk volume (V), 18 d high-intensity (I), and 10 d unloading (U) . Maximal oxygen uptake ($\dot{V}O_{2max}$), power output at ventilatory threshold (POT_{vent}), cycling economy, and time to complete 20 km on a computerized roller system were evaluated before and after each phase. No significant changes were observed during baseline. $\dot{V}O_{2max}$, POT_{vent}, and cycling economy increased ($p<0.05$) following V, with no further significant changes after I or U. Time trial performance improved ($p<0.05$) following V, and at U compared to V. Lymphocyte subsets, IL-1β, IL-6, TNFα, cortisol, and testosterone did not change significantly at V, I, or U as compared to baseline. It was concluded that the endurance training did not cause immunosuppression as measured by these parameters.

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Chapter 1

Introduction

1.1 Introduction to the Problem

There is a common belief that moderate amounts of exercise may decrease the risk of infection through stimulation of the immune system, whereas intense exercise can cause immune deficiency and thus an increased susceptibility to infection (1, 2, 3, 4). It is believed by some that endurance athletes who undergo intense, heavy training have an increased susceptibility to illness (2). Additionally, the severe exertion of competition itself has been reported to increase the risk of an upper respiratory tract infection in athletes (5). This thesis has attempted to address the relationship between endurance training stress and immunity.

Adrenal secretion of corticosteroids may play an important role in the relationship between heavy training stress and immune suppression (6). It is well known that organisms under stress produce and secrete elevated amounts of adrenal steroids (7) which can suppress immune function (8), but the causal relationship between chronic stress, particularly physical stress in the form of heavy endurance exercise, and immunosuppression has not been conclusively determined. Adrenal steroid concentrations, in particular cortisol, have been measured in blood (9, 10) and urine (11) as estimators of the magnitude of training stress, but the response of resting cortisol to heavy endurance training, has not been thoroughly investigated. Additionally, researchers have not clearly defined the effects of chronic psychological stress on resting blood cortisol concentrations (12). Cortisol is secreted in response to an acute stressor, and the main function is to mobilize fuel for the fight-or-flight response by stimulating glycogenolysis and lipolysis (13). It could be theorized that if an individual was chronically stressed, cortisol production could remain greater than normal for as long as the stressor is present. This activation of the hypothalamic-pituitary-adrenal axis and subsequent increase in glucocorticoid production has been shown to occur in parallel with thymic and lymphoid shrinkage (7). The potent immunomodulating effects of corticosteroids have been established (8). This has been supported by the widespread use of glucocorticoids for treatment of allergy, inflammation, and autoimmune disease (14).

Reproductive function may also be suppressed by chronic stress (15) as indicated by the suppressive actions of glucocorticoids on the production of testosterone (16) and on other reproductive function (6, 17). Decreased testosterone concentrations have been found in heavily training subjects (18, 19, 20), and it has been proposed that resting testosterone concentrations may be inversely related to the magnitude of training stress.

Athletes may provide an unique opportunity to research the effects of chronic stress. Competitive athletes regularly train at heavy exercise loads in an attempt to maximize training benefits, and in extreme cases this may cause overtraining. Some of the signs and symptoms of the overtraining syndrome have been chronic, high levels of fatigue, stagnancy or deterioration in performance, and mood changes (21, 22, 23). The immune system in overtrained athletes may be suppressed (21), however in the absence of overtraining, the resting immune status of athletes has been reported to be normal (24). The importance of recognizing the signs and/or symptoms of an overtraining syndrome is paramount for an athlete. In a longitudinal training study conducted to induce an overtraining syndrome in experienced endurance runners, it was reported that the subjects could not reproduce their former personal best performances during the following competitive season (25). Therefore, it is important to research the physiological changes that occur in an athlete while they undergo heavy endurance training.

Because of the health risks, it is generally considered unethical to conduct research that causes an overtraining syndrome. Therefore, research designs that do not negatively impact subject health must be proposed. Recognizing the onset of an overtraining syndrome may be possible by documenting the signs and symptoms of stressful training states that precede the overtraining syndrome. There is a continuum of exercise stress ranging from gentle exercise to "overtraining syndrome". In their reviews, Kuiper and Keizer (22) and Fry et al. (21) have defined the terminology used to describe the various physical states along this continuum. Their definitions include the following:

Overload training - a practice of training which involves stressing an individual to provide a stimulus for adaptation and supercompensation (a state of heightened work capacity).

Training fatigue - the fatigue that is experienced following heavy overload training and/or restricted rest.

Overtraining - a general term describing a training process leading to a decreased performance capacity, despite increased or maintenance of training load.

Overreaching - is short term overtraining, that can be reversed by longer than normal (1 - 2 weeks) regeneration period.

Overtraining syndrome - is the state of chronically depressed performance accompanied by symptoms that may be psychological, immunological, hematological, endocrinological, neural, and/or biochemical. Recovery from overtraining syndrome may be reversed only after an extensive period (months) of rest. However, there are no studies charting the progress of the overtraining syndrome. Therefore, it is not known exactly what lasting effects the overtraining syndrome may have.

Because overreaching occurs before overtraining, recognizing the initial signs of overreaching may aid in eliminating the possibility of inducing an overtraining syndrome. Understanding the physiology accompanying overload training may aid in determining the factors to monitor in order to recognize the phenomena of overreaching.

1.2 Statement of the Problem

Athletes, over the span of their careers, frequently push themselves into overreaching states as a result of overload training and/or inadequate recovery. It has been suggested that the immune system of an overreached or overtrained athlete may be compromised. Whether or not endurance training has an altering effect on the immune system has yet to be determined. If immune function can be suppressed by endurance training, it would be important to know the training intensity, frequency, and duration over which such a suppression may be realized, so that the training stress could be reduced and immune suppression could be halted.

In the interest of athlete health, it is important to determine the physiological, endocrinological, and immunological changes that occur during training in an attempt to obtain a better understanding of the physiological implications of training, overreaching, and overtraining. In order to study the overtraining phenomenon ethically, the stages of training stress and fatigue which precede the overtraining syndrome, such as overload training and overreaching, should be investigated to determine the possible physiological implications for the health of the athlete.

1.3 Purpose

The purpose of this study was to examine the changes in selected immunological, endocrinological, and performance variables over nine weeks of endurance cycle training. The program consisted of continuous training (at selected intensities around ventilatory threshold) followed by 18 days of interval training (at $\dot{V}O_{2\max}$), and finally, 10 days of recovery (reduced volume) training. Dependent variables included resting counts of lymphocyte subsets ($CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^-CD16^+CD56^+$, and $CD19^+$) and serum cytokines (IL-1β, IL-6, and TNFα); resting urinary cortisol and serum testosterone; and, maximal oxygen consumption ($\dot{V}O_{2\max}$), power output at ventilatory threshold (POT_{vent}), cycling economy, and time to complete a 20 km performance time trial on an indoor roller system.

1.4 Hypothesis

Overload endurance training will produce a stress response characterized by elevated adrenal cortisol secretion, as shown by elevated 24 hour urinary free cortisol concentration; a suppressed reproductive function, characterized by decreased serum testosterone concentrations; and, suppressed immune function, characterized by altered circulating lymphocyte subset counts and cytokine concentrations. Both ventilatory threshold intensity endurance cycling and $\dot{V}O_{2\,max}$ intensity interval cycling will produce a stress response. An "unloading" period of reduced training volume will allow recovery from training fatigue.

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Chapter 2

Review of Literature

2.1 Introduction

There is a general belief that a training program consisting of moderate exercise has an immunoenhancing effect, whereas strenuous exercise and athletic competition may suppress immune function, thereby increasing susceptibility to infection (1, 2, 3). In support of the idea that moderate exercise enhances the ability of the immune system to combat and eradicate foreign pathogens, it has been concluded that regular exercise may improve immunity against upper respiratory tract infection (URTI) (4). This conclusion was based on the findings of a study that found that the duration of URTI symptoms were decreased from an average of 11 days per URTI to 5 days per URTI in 36 mildly obese women, ages 25 to 45, who exercised for 15 weeks by walking briskly for 45 minutes, five days per week when compared to non-exercising controls (4). In support of the idea that strenuous exercise may hinder the ability of an immune system to respond to an antigenic challenge, it has been suggested that running dosage (mileage) was a significant risk factor for upper respiratory tract infections (URTI) (5). The authors reported that a greater risk of URTI occurred with a running mileage averaging 2.4 - 3.9 miles per day over 12 months compared to 1.3 - 2.4 miles per day over 12 months (5). There seems to be a relationship between exercise and immunocompetence, although the actual mechanisms by which the two interact are not well understood.

This review will first address the topic of acute endurance exercise effects on immune cell counts. It will then review the effects of chronic endurance training on immune cell counts. Inquiry into a potential mechanism by which endurance training and the immune system interact will follow. The stress response, brought on by heavy training stress, and the associated humoral modulators will be discussed because the potential immunomodulating effects are of interest.

2.2 Immune Responses to Endurance Exercise

2.2.1 Immune Cell Count Responses to Acute Endurance Exercise

It has been established that there is a biphasic alteration in circulating blood leukocyte counts during and after acute high-intensity aerobic exercise (6, 7, 8, 9). During exercise, there was an exercise-induced leukocytosis, granulocytosis, lymphocytosis, and monocytosis. During immediate recovery (up to 2 hours) after exercise, leukocytosis, granulocytosis, and monocytosis remained, but total lymphocyte counts fell to levels significantly below pre-exercise levels (6, 7).

To give examples of the magnitude that cell counts change, results from three papers in this field of study will be presented.

2.2.1.1 Leukocytes

Nieman et al. (8) reported that peripheral blood total leukocyte count started rising one hour into exercise and rose to 178% above pre-exercise baseline values by 1.5 hours into recovery. The exercise load imposed was a three hour run at marathon race pace on a treadmill. The subjects chosen were ten (nine male and one female) experienced (8.9 ± 1.6 years of running experience), fit (running an average of 67.3 ± 7.7 km/week) marathon runners. Shek et al. (6) reported that within 30 min after exercise, total leukocyte count had risen significantly by 50%. The exercise load imposed was treadmill running at 65% of $\dot{V}O_{2max}$ for 120 min or until core temperature reached 40°C. Six fit ($\dot{V}O_{2max}$ = 66.5 ± 5.3 ml/kg/min) male volunteers served as subjects. Shinkai et al. (7) reported that total leukocyte count increased, during exercise, to 59% above pre-exercise baseline values by the end of exercise. Following exercise, leukocyte count continued to rise and was measured at 2.3 times baseline values by 120 min of recovery. The exercise load imposed was 60 min of cycling at 60% of $\dot{V}O_{2max}$. Subjects were 21 young male volunteers who, although they did not exercise regularly, were fit enough to undertake a maximal cycle-ergometer test. In summary, total leukocyte count increased during exercise, and remained elevated or continued to increase during recovery.

2.2.1.2 Granulocytes

Of the total leukocyte population, granulocytes make up approximately 60% (10). The response kinetics of granulocytes were similar to that of total leukocytes. Nieman et al. (8) reported that total granulocyte count was 235%, 291%, and 197% above pre-exercise baseline counts at 5 min, 1.5 h, and 6 h of recovery respectively. These authors reported that this rise was primarily due to a rise in neutrophils. Neutrophils contribute approximately 94% of granulocytes, eosinophils contribute 5%, and basophils contribute 0.9% (10). Basophils did not change significantly during or following a 3 h run. Eosinophil counts significantly fell from contributing 2.9% of all granulocytes before exercise to 0.5% at 1 h of exercise. No eosinophils were detected in all but three of the ten subjects at 1 h of exercise. By 21 h of recovery, eosinophils had returned to contributing 1.8% of the total granulocytes, which was still significantly depressed when compared to pre-exercise values. Shek et al. (6) reported that total granulocytes rose to

2.5 times their pre-exercise values. The increase in granulocyte counts rose in a pattern parallel to the increase in total leukocyte counts. Shinkai et al. (7) reported that total granulocyte count had risen 59% above pre-exercise values by the end of exercise. At 120 min of recovery, granulocyte count had risen to 2.3 times above pre-exercise values. In summary, neutrophils accounted for the majority of granulocytes. Circulating granulocyte counts increased by as much as 200% during heavy acute endurance exercise, and continued to increase during recovery from exercise.

2.2.1.3 Monocytes

Nieman et al. (8) reported that monocytes were significantly increased by 67% and 79% at 5 min and 1.5 h of recovery respectively. Shek et al. (6) reported that monocytes had risen 100% by the end of exercise and had remained elevated for at least 120 min into recovery. Shinkai et al. (7) reported that monocytes were elevated 66% above baseline at the end of exercise and had returned to baseline levels by 30 min into recovery. In summary, circulating monocyte counts increased following an acute exercise bout by 60 - 100%. Monocyte counts remained elevated for a short time (30 - 120 min) following exercise.

2.2.1.4 Total Lymphocytes

Nieman et al. (8) reported that total lymphocyte count significantly increased by 31% at 1 h of exercise but then decreased to 19% below pre-exercise values by 1.5 h of recovery. Shek et al. (6) reported that total lymphocyte count rose to a level 1.8 times that of the pre-exercise resting levels within the first 30 min of the 120 min endurance exercise bout. At 30 min of recovery, counts had dropped to pre-exercise levels. At 60 and 120 min into recovery, total lymphocyte count had dropped to 30% below pre-exercise values. Shinkai et al. (7) reported that total lymphocyte count had risen to 56% above baseline at the end of exercise and had dropped significantly below baseline levels by 30 and 120 min of recovery. In summary, circulating total lymphocyte counts increased during acute endurance exercise, but then decreased to below pre-exercise values immediately following exercise.

2.2.1.5 Total T ($CD3^+$) Lymphocytes

Nieman et al. (8) reported that there were no significant changes in total T cell count. Shek et al. (6) reported that exercise triggered an initial significant increase of a peak 58% above pre-exercise counts at 30 min of exercise. Total T cell count remained elevated throughout the 120 min exercise time, declined to pre-exercise levels by 30 min into recovery and then declined significantly to 42% below pre-exercise values by 2 h into recovery. Shinkai et al. (7) reported that total T cell count was significantly elevated above baseline during exercise and then significantly depressed below baseline (77% of pre-exercise baseline values) during recovery. The response of circulating counts of total T cells to acute endurance exercise was reported to have not significantly changed (8) or to have significantly increased during exercise and then significantly decreased to below pre-exercise values immediately following exercise (6, 7).

2.2.1.6 T_{helper} ($CD4^+$) Lymphocytes

Nieman et al. (8) reported that there was no significant change in the T_{helper} cell count. Shek et al. (6) reported that T_{helper} cell count rose significantly (approximately 40%) during exercise and then dropped, but not significantly so, below pre-exercise values during recovery. Shinkai et al. (7) reported a significant 16% rise in T_{helper} cell count during exercise. During recovery, T_{helper} cell count dropped significantly below pre-exercise values. At 120 min into recovery, T_{helper} cell count was 80% of pre-exercise values. The response of circulating T_{helper} cell counts to acute endurance exercise are variable, although there seems to be a trend of an increase during exercise and a decrease below pre-exercise values following exercise.

2.2.1.7 $T_{cytolytic}$ ($CD8^+$) Lymphocytes

Nieman et al. (8) reported a result of no significant rise in $T_{suppressor/cytolytic}$ cell count during exercise, although there was a non-significant rise. Significant decreases at 1.5, 6, and 21 hours of recovery were reported. Shek et al. (6) reported that $T_{cytolytic}$ cell count rose significantly during exercise (approximately 70% above pre-exercise values by 30 min) and then fell significantly below pre-exercise values (approximately 80% of pre-exercise values) during recovery. Skinkai et al. (7) reported that $T_{cytolytic}$ cell count had risen significantly (43%) above pre-exercise values by the end of exercise, and then had fallen significantly to 78% of pre-exercise values by 120 min of recovery. The response of circulating $T_{cytolytic}$ cell counts to acute endurance exercise have been reported to be similar to the response of T_{helper} cells, although the magnitude of change (increase during exercise and decrease to below pre-exercise values during recovery) in counts may be greater in $T_{cytolytic}$ cells.

2.2.1.8 $T_{helper}/T_{suppressor/cytolytic}$ ($CD4^+/CD8^+$) Ratio

Nieman et al. (8) reported that the $CD4^+/CD8^+$ ratio was significantly elevated (39% above pre-exercise values) at both 1.5 h and 21 h of recovery. Shek et al. (6)

reported that the CD4$^+$/CD8$^+$ ratio followed a downward trend during the exercise period, but followed an upward trend during recovery. The downward trend ratio values were significantly below pre-exercise values, and the recovery ratio values had returned to values not significantly different than pre-exercise values. Shinkai et al. (7) reported a similar downward trend in CD4$^+$/CD8$^+$ ratio values during exercise that were followed by an upward trend back to pre-exercise values during recovery. Neither of these changes were significant. In summary, the CD4$^+$/CD8$^+$ ratio appears to decrease during exercise and then increase again during recovery from exercise.

2.2.1.9 B (CD19$^+$) Lymphocytes

Nieman et al. (8) reported that B cell count rose significantly to 17% and 42% above pre-exercise values at 5 min and 6 h into recovery, respectively. Shek et al. (6) reported that there was no significant change in B cell count, although B cell count did show a non-significant trend of increasing during exercise and decreasing below pre-exercise values during recovery. Shinkai et al. (7) reported that B cell count rose significantly to 17% above pre-exercise values at the end of exercise, and then fell back to pre-exercise values by 30 min into recovery. B cell count stayed around the pre-exercise value for the duration of the 120 min recovery. Circulating B cell counts have been reported to rise during exercise, and to remain elevated during recovery, or to return to pre-exercise values during recovery.

2.2.1.10 Natural Killer (CD3$^-$,16$^+$, 56$^+$) Lymphocytes

Shek et al. (6) reported a significant rise of 40 - 75% in NK cell count during exercise. During recovery, NK cell count fell significantly to levels 40 - 60% below pre-exercise values. It was reported that NK cell count remained depressed by 40% below baseline for at least 7 days. Shinkai et al. (7) reported that NK cell count rose significantly to 169% above pre-exercise values by the end of exercise. During recovery, NK cell count fell significantly to 54% of pre-exercise values by 120 min of recovery. Nieman et al. (8) did not report natural killer cell results. Natural killer cells showed the largest magnitude biphasic response of all of the lymphocyte subsets

2.2.1.11 Summary of Acute Exercise Effects on Immune Cell Counts

Total blood leukocyte counts rose significantly during and after moderate- to high-intensity endurance aerobic exercise. Of the leukocyte types, granulocytes demonstrated the largest percent increase in number (150 - 300%), monocytes the second largest percent increase (65 - 100%), and lymphocytes the third (30 - 60%). Of the total leukocyte population, granulocytes contribute 34.6 - 81% (95% range) of total cell numbers, lymphocytes contribute 19.6 - 52.7%, and monocytes contribute 2.4 - 11.8% (10). Lymphocytes demonstrated a distinct biphasic response that neither granulocytes or monocytes showed. Lymphocyte counts increased during exercise and then decreased to below pre-exercise values after exercise. The lymphocyte subset which showed the greatest percent changes in blood count was the natural killer cell (increased by 40 - 169% during exercise and decreased to 40 - 60% below pre-exercise values during recovery). $T_{cytolytic}$ and T_{helper} cell counts demonstrated a biphasic response to acute endurance exercise, although the magnitude of change was less than that of natural killer cells. One study (8) reported that $T_{cytolytic}$ and T_{helper} cells did not change significantly at all. B cells were reported to increase during exercise and to remain elevated immediately following exercise, although the results from studies are not all in agreement. The normal resting percentage appearance of lymphocyte subsets in blood is that natural killer cells contribute ~10% of total lymphocytes, $T_{cytolytic}$ cells represent 20 - 25%, T_{helper} cells represent 50 - 60%, and B cells represent 10 - 15% (10).

2.2.1.12 Mechanism Explaining the Alteration in Leukocyte Counts

An explanation for this transient increase in circulating leukocytes during acute exercise is that acute exercise stimulates the release of adrenaline (8, 11), and adrenaline stimulates the demargination of leukocytes from blood vessel walls (12, 13). Lymphocyte cell surface concentrations of β-adrenoreceptors have been shown to increase during exercise (14). β_2-adrenergic stimulation has been shown to cause a detachment of natural killer cells from the marginated pool on blood vessel endothelium (15, 16). β-blockade by the β-adrenoreceptor antagonist propranolol has been shown to significantly inhibit natural killer cell blood count increases when stimulated by an acute emotional stressor (a first-time tandem parachute jump) (17). A proposed mechanism as to how these large transient changes in circulating concentrations of leukocytes occur has been that exercise acts to alter lymphocyte expression of cell-surface adhesion molecules (18, 19). Lymphocyte cell surface expression of the adhesion molecules of L-selectin (CD62L) (19) and lymphocyte function-associated antigen-1 (LFA-1 or CD11a) (18, 19) may play roles in influencing the mobilization of granulocytes and lymphocytes in and out of circulation, during and after exercise. L-selectin is a cell-surface adhesion molecule that plays an important role in the homing of naïve lymphocytes (10). "Homing" refers to the action of cells obtaining tissue-specific (e.g. endothelial cells) attachment (10). LFA-1 is another cell surface adhesion molecule that plays an important role in the homing of leukocytes to inflamed tissue (18). LFA-1 is expressed on more than 90% of thymocytes, mature T cells, B cells, granulocytes, and monocytes (10). In summary, exercise-induced β-adrenoreceptor stimulation may alter cell-surface adhesion molecule expression rates, thus influencing the rate of detachment and/or reattachment of circulating leukocytes to surrounding endothelium (19).

The late onset response of cortisol to aerobic exercise of moderate to high intensity has also been suggested to play a role in the disappearance of circulating cells from the blood compartment (8, 12).

2.2.1.13 Effect of Acute Exercise-caused Redistribution of Leukocyte Count Changes on Immunity

Acute exercise does cause alterations in circulating leukocyte counts. It has not been determined what effects this redistribution of counts and ratios of leukocyte types has on functional immunity. To predict or research this question of altered immunity, a thorough understanding of how immune cells circulate through an organism and how they move into different types of tissues is necessary. Such knowledge will aid in understanding how such large transient changes in circulating leukocyte counts occur, and thus will be important when making assumptions about immune competence levels based on changes in blood lymphocyte cell counts. When only ~0.2% of the total lymphocyte mass circulates in the blood at one time (20), or when an increase of 150% in circulating granulocytes means that the percent of total neutrophils (~94% of total granulocytes (10)) circulating in blood has perhaps risen from 3% (21) to approximately

7%, understanding the context in which circulating immune cell counts change is a necessity in order to draw valid conclusions regarding alterations in immunocompetence.

Considering that acute exercise does cause an alteration of immune cell counts, it is feasible that repeated bouts of acute exercise, may cause a chronic alteration in immune cell counts and/or function. There have been few longitudinal training studies that have measured immune cell counts in closely monitored, heavily training endurance athletes. The next section of this review will focus on longitudinal training studies, and some cross-sectional studies, which have measured immune cell counts in endurance training subjects.

2.2.2 Lymphocyte Subset Count Responses to Endurance Training

There have been many papers that have studied immunological responses to acute exercise. Two review articles (22, 23) have summarized the results of current papers. Fewer reports have described the influence of chronic heavy endurance training on the immune system. A few cross-sectional reports have compared athletes to non-athletes (24, 25, 26, 27) and even fewer longitudinal studies have followed athletes through a training regime (28, 29, 30). Such studies are time consuming, expensive, and labour intensive, but they have the potential to contribute much to an understanding of modulation of the immune system caused by heavy endurance training. The following section of this literature review will address the responses of testing immunological parameters to endurance training. This section will highlight findings of cross-sectional studies, short term (<3 weeks of imposed heavy training load) longitudinal studies, and long term (>3 weeks of imposed heavy training load) longitudinal studies.

2.2.2.1 Cross-sectional Studies

Lewicki et al. (27) conducted a cross-sectional study in which 20 well conditioned healthy male competitive cyclists, aged 20.9 ± 2.0 years, were compared to 19 healthy male physical education students, aged 18.9 ± 0.7 years. The cyclists had cycled, on average, 20,000 km/year for the past five years, whereas the control group's exercise history was limited to physical activity classes in secondary school.

Lewicki et al. (27) measured a variety of immune cell counts and activities in these subjects before, immediately (3 min) after, and 2 hours after a maximal exercise test. Resting absolute and percentages of neutrophils, eosinophils, and monocytes did not differ between groups. Total leukocyte, neutrophil, and monocyte adherence was significantly lower in the athletes than in controls. Total lymphocyte values were not reported. Adherence was measured by calculating the percentage of cells that had filtered through a 15 mm long Pasteur pipette column filled with 50 mg of nylon wool and compared to the cell count in the original sample. Adherent properties of cells are mediated by cell surface receptors and are important for attachment to endothelial walls in order to allow migration to sites of infection in tissues (10). Neutrophil bactericidal activity, measured as a percent of viable intracellular bacteria, was significantly lower in athletes than in controls.

Rowbottom et al. (25) measured a variety of physiological, biochemical, immunological, haematological, and psychological parameters in ten athletes diagnosed as suffering from overtraining syndrome (OTS). These results were reported to be compared to control, age-matched normal range values obtained from the Department of

Clinical Haematology and the Department of Clinical Biochemistry at the University of Western Australia, and from published literature. Normal ranges were considered as being between the 5th and 95th percentiles of the normal distribution. Mean total white blood cell, neutrophil, eosinophil, basophil, total lymphocyte, and monocyte counts were all within normal ranges in the OTS subjects. There were no significant differences between the normal values for an age-matched subject group and the mean values for the ten subjects for any of the lymphocyte subset parameters measured. The lymphocyte subset parameters measured were total T ($CD3^+$) cells, T_{helper} ($CD4^+$) cells, $T_{cytolytic}$ ($CD8^+$) cells, B ($CD19^+$) cells, natural killer (NK) ($CD56^+$) cells, %$CD25^+$ (IL-2 receptor α chain; i.e. indicative of prior lymphocyte activation), %$HLA\text{-}DR^+$ (Human Leukocyte Antigen, DR allele gene product - percent of T cells which express a class II major histocompatability complex (MHC) receptor; i.e. indicative of prior T cell activation), and $CD4^+/CD8^+$ ratio. There was one significantly different blood parameter between the two groups. Plasma glutamine was lower in the OTS than in the control group. Glutamine is an amino acid which has been shown to be a key substrate for cells of the immune system, essential as a precursor for nucleotide biosynthesis and as a major energy source (31).

Nieman et al. (26) compared immune function of 18 young male adults with at least 3 years of competitive experience in running or bicycling, and a $\dot{V}O_{2max}$ greater than 60 $ml \cdot kg \cdot min^{-1}$, to eleven untrained male adults (less than 3 aerobic sessions per week during the previous year). Concentrations of circulating leukocyte and lymphocyte subsets (NK ($CD3^-CD16^+CD56^+$) cells, B ($CD20^+$) cells, total T ($CD3^+$) cells, activated T

($CD3^+HLA\text{-}DR^+$) cells) were not significantly different between groups. Natural killer cell cytotoxic activity, measured using the chromium release assay (26), and T cell function, measured as Concanavalin A- (Con A) induced lymphocyte proliferation, were not significantly different between groups, whether expressed unadjusted or adjusted on a per-cell basis.

From reviewing these cross-sectional studies, it appears that leukocyte and lymphocyte subset counts are not different in athletes as compared to controls. Lewicki et al. (27) reported some significant differences in immune cell function in athletes as compared to controls, but cell activity measurements performed by Rowbottom et al. (25) and Nieman et al. (26) did not support this finding.

If there were any fundamental basic differences in immune cell count profiles between athletes and sedentary or moderately active people, cross-sectional studies would be effective research tools to determine these differences. These studies did not demonstrate any significant differences in immune cell counts between athletes and non-athletes, although definitive conclusions cannot be formed from the results of only three studies. When comparing these cross-sectional studies, differences in training protocols, time since last exercise session, and conditions prior to sample collection were factors that were not always controlled for and were not necessarily similar between studies. Longitudinal studies generally attempt to control for these variables and thus offer potentially greater reliability and validity in research design. A review of several longitudinal studies follows.

2.2.2.2 Immune Responses to Short Term (< 3 weeks) Chronic Heavy Endurance Exercise

Fry et al. (32) conducted a study in which five subjects undertook ten days of intensive twice daily interval training followed by five days of active recovery. Five male (aged 31.6, standard error of the mean (SEM) = 3.5) experienced, fit runners served as subjects. Interval sessions consisted of 15 × 1-minute running intervals at either 18 $km \cdot h^{-1}$, 19 $km \cdot h^{-1}$, 20 $km \cdot h^{-1}$, or 21 $km \cdot h^{-1}$ at a treadmill grade of 1%. There was no control group. Immune parameters were monitored on the first day of training, prior to any exercise (day 1); on day 6 and on day 11, after five and ten days of twice daily interval training respectively; and on day 16, after five days of active rest. Blood samples, in which immune parameters were assayed, were obtained at 6:00 a.m. on each of these four test days. Results indicated that there were no significant changes in total leukocyte count or any of the leukocyte subset counts over the course of the study. Some of the lymphocyte subset counts were significantly altered when pre-training (day 1) values were compared to during- or post-training measurements. Natural killer ($CD56^+$) cell count fell significantly from pre-exercise (day 1) levels to after six days of twice daily interval training (day 6), and remained depressed through the completion of ten days of interval training (day 11) and after five days of active rest (day 16). The authors felt that there was an increase in the level of activation of peripheral blood lymphocytes as indicated by significant elevations in the expression of $CD25^+$ and $HLA\text{-}DR^+$ cell-surface molecules, as well as a significant depression of the $CD3^+/CD25^+$ ratio at day 6, day 11, and day 16. It was felt that the rise in $CD25^+$, which is the IL-2 receptor α-

chain, signified recent host cell activation, and the fall in $CD3^+/CD25^+$ ratio indicated that the percentage of T cells with IL-2 receptors on their membranes had significantly increased, thus a greater percentage of the available T cells had been activated. The increase in cells displaying $HLA\text{-}DR^+$ cell surface molecules, components of class II MHC, indicated that these cells had been involved in T_{helper} cell activation (10). The lymphocyte subsets that were not significantly altered were total T ($CD3^+$) cells, B ($CD20^+$) cells, T_{helper} ($CD4^+$) cells, $T_{cytolytic}$ ($CD8^+$) cells, and $CD4^+/CD8^+$ ratio.

The next two studies to be reviewed (33, 34) measured immune variables over two separate ten day phases of increased training stress. Pizza et al. (33) conducted a study to examine the effect of a 200% increase in training volume on circulating leukocyte and lymphocyte subsets, and to compare the training modes of running-only and running and cycling-both as to their effects on circulating immune cell counts. The subjects were 11 competitive male runners who had at least one year of racing experience in 10 to 42 km long running events, a 10 km performance time of less than 38 min, and current training distances greater than 50 $km\cdot wk^{-1}$ and less than 80 $km\cdot wk^{-1}$. The research design training protocol consisted of 30 days of "normal training" (NT), in which training was not prescribed by the investigators and subjects maintained distance and intensity logs of their personal training. The second phase lasted 14 days and consisted of training at 80% of NT. The third phase was 10 days at 200% of NT. The fourth phase was identical to phase two. The fifth phase was similar to phase three, except that the training mode, either run-only or run and bike-both, was reversed to that performed in phase three. Blood samples were obtained following the 30 day NT phase, prior to (day 0), mid-way (day 5), and following (day 11) each increased (+200%) training phase (phases three and five).

Pizza et al. (33) reported that there were no significant changes in any cell counts, when comparing results from the end of NT to the end of reduced training (80% NT). There were no significant differences due to training mode (i.e. run-only vs. run and bike-both). Total lymphocyte counts fell significantly from day 0 to day 11 of both increased (200% NT) training phases (phases three and five), while total leukocytes, neutrophils, and monocytes did not change significantly. Total T cell ($CD3^+$) count fell significantly (-14.6% and -15.6%) during run and bike-both and run-only phases, respectively, from day 0 to day 11. T_{helper} ($CD4^+$) cell count fell significantly (-12.0% and -14.8%) during run and bike-both and run-only phases, respectively, from day 0 to day 11. $T_{cytolytic}$ ($CD8^+$) cell count fell significantly (-7.7% and -23.2%) during run and bike-both and run-only phases, respectively, from day 0 to day 5, and also when comparing day 0 to day 11 (-17.9% and -13.9%) during run and bike-both and run-only phases, respectively. A result of no significant change was reported for natural killer ($CD16^+$) cells. Two possible reasons, given by the authors, for the reductions in T lymphocytes ($CD4^+$ and $CD8^+$) were that T cells migrated into damaged muscle from blood, and that the acute exercise effort performed on day 10 could have had an effect on immune cell counts since blood samples were obtained only 13 to 16 h after the workout. The authors did note that the reduction in circulating T lymphocytes was not associated with cortisol, adrenocorticotrophic hormone (ACTH), free and total testosterone, or global mood score. No infectious episodes accompanied these depressions in $CD4^+$ and $CD8^+$ lymphocyte counts.

Kajiura et al. (34) conducted a study which was divided into four phases of alternating easier and heavier endurance training. Twelve male (18 - 23 yr.) recreational and competitive runners (mean $\dot{V}O_{2max} = 63.35 \pm 4.75$ ml·kg^{-1}·min^{-1}, normal training distance = 35.45 ± 9.85 km/wk) served as subjects. The study training regimen consisted of four 10-day phases of varying volume and intensity training prescription. Each of the four phases was ten days in length. Phase 1 and phase 3 were "baseline" phases of low-volume/low-intensity running (LV/LI) at an estimated intensity of 60 - 70% $\dot{V}O_{2max}$. Phase 2 and phase 4 were either high-volume/high-intensity (HV/HI) or high-volume/low-intensity (HV/LI) running. For the HV/LI training phases, running distance was increased by 100%. For the HV/HI phases, running distance was increased by 100% and on alternate days, a series of high intensity (95 - 100% $\dot{V}O_{2max}$) 1000 m intervals were performed. Subjects were assigned to one of two different cross-over training group orders: (1) LV/LI, HV/LI, LV/LI, HV/VI or (2) LV/LI, HV/VI, LV/LI, HV/LI. Resting blood samples, taken prior to exercise, were obtained on three occasions during each phase: on days 1, 4, and 7. Subjects were requested to not exercise during the 12 hours prior to blood sampling. Kajiura et al. (34) presented no leukocyte count data, and chose to present the lymphocyte subset count data as percentages of total lymphocytes. Resting percentages of total T ($CD3^+$) cells, T_{helper} ($CD4^+$) cells, $T_{cytolytic}$ ($CD8^+$) cells, and $CD4^+/CD8^+$ values did not change significantly over the four training phases. Counts of HLA-DR^+ and immunoglobulins G, A, and M did not change significantly over the four training phases. Of interest, baseline cortisol levels did not change significantly over the four training phases. It was concluded that repeated intensive exercise within the space of a few days does not appear to suppress resting immune status.

Verde et al. (35) studied lymphocyte counts and activities over a three week period of 38% increased training volume in ten elite male distance runners (age 29.8 ± 1.7 yr; $VO_{2max} = 65.3 \pm 4.9$ ml·kg·min^{-1}; 10 km time = 31 min 43 s ± 1 min 46 s). The training program consisted of three weeks of baseline training (B1), three weeks of heavy training (HT), and three weeks of baseline training (B2) again. HT was defined as a 38% increase in training volume over baseline training load. Blood samples were obtained at the end of each three week phase. Training was limited to short distance and low intensity work in the 36 hours immediately preceeding blood sample collection.

Verde et al. (35) represented the lymphocyte data as percentages. Total T lymphocytes, as a percentage of total leukocytes, was significantly lower following the second baseline training phase (B2) than following the first baseline training phase (B1) and the heavy training (HT) phase. There were no significant differences between training phases in B cells as a percentage of total lymphocytes. T_{helper} cell percentage was significantly higher following B1 compared to following HT and B2, and was significantly lower following B2 compared to following B1 and HT. Thus, T_{helper} cell percentage fell consecutively over all three training phases. $T_{cytolytic}$ cell percentage was significantly elevated following the HT phase compared to both baseline phases, and was significantly lower following B2 than following both B1 and HT. $CD4^+/CD8^+$ ratio was significantly lower following B1 and HT than following B2. Cell-mediated immune function was assessed by measuring mitogen-induced cell proliferation. Phytohemagglutinin (PHA) and concanavalin-A (ConA) were utilized to specifically stimulate T cells. PHA-stimulated T cell proliferation rates were non-significantly elevated following HT (+24.6%) compared to following B1. ConA-stimulated T cell proliferation rates were significantly higher following HT (+32.2%) compared to following B1. Following B2, the elevated proliferative response remained significantly higher than B1. B cell immunoglobulin synthesis rates were measured as indicators of humoral immune function. Pokeweed mitogen-induced IgG and IgM synthesis were significantly lower following HT compared to following B2.

All four of these studies measured immune parameters in elite endurance athletes. The increased training load was imposed for a short duration, and allowed a large increase in training load. These studies gave conflicting results. Fry et al. (32) reported no changes in circulating lymphocyte counts with the exception of a significant decrease in natural killer cell counts in athletes following five days of twice daily interval (VO_{2max}) training. Pizza et al. (33) reported significant decreases in T cell counts (total T, $T_{cytolytic}$, and T_{helper} cells) as the result of a 10-day period of 200% increased training volume, but no significant change in natural killer cell counts. The results of these two studies suggest that the imposition of a markedly increased training load on endurance athletes may affect the circulating immune cell counts of these athletes. Comparing results of absolute cell counts to cell counts as a percentage of total cells is difficult and may be misleading. A decrease in the percentage of a certain cell type does not necessarily mean that there are fewer of that type of cell. The methods by which researchers have reported their data have not been standardized. The optimal way to present immune data to maximize relevance to immunocompetence has not been determined by researchers in the field of exercise physiology, and standardization is lacking.

Changes in immune cell activation parameters were more significant than changes in immune cell counts or percentages reported in these studies. Although the results were conflicting, it does seem that there is some alteration to immune function as a result of short term heavy endurance training.

2.2.2.3 Immune Responses to Long Term (> 3 weeks) Heavy Endurance Training

Baum et al. (28) conducted a longitudinal training study that followed several immune parameters in 20 track and field runners of regional, national, and international rank over approximately four months of training and competing. The training protocol was divided into three phases: (phase 1) two months of endurance training consisting of almost daily endurance runs of 12 to 25 km per session with a weekly distance between 60 and 160 km; (phase 2) eight weeks of more intensive training consisting of endurance runs and anaerobic interval runs that caused lactate concentrations to reach 15 mmol/l; and (phase 3) a competitive period in which the increased psychological stress of competition was added to a heavy training workload. A weakness of this paper was that it did not indicate how long this period was. Thirteen non-athletes and leisure time athletes served as a control group. A further weakness of this study was that the control group underwent all of the testing procedures only once at an undisclosed point in the protocol.

Baum et al. (28) reported that there were no changes in total leukocyte or total lymphocyte counts. Total monocyte count was significantly higher after the competition phase of training (phase 3) as compared to after endurance training (phase 1). When comparing athlete subject values between the three training phases, no significant differences were found in any of the lymphocyte subset counts (total T ($CD3^+$) cells, total B ($CD20^+$) cells, T_{helper} ($CD4^+$) cells, $T_{suppresser}$ ($CD8^+$) cells, and natural killer ($CD16/56^+/CD3^-$) cells). However, no pre-training values were obtained for any of these parameters. When compared to the controls, $T_{cytotoxic}$ ($CD16/56^+/CD3^+$) cell counts were significantly higher in athletes after the endurance training period (phase 1) and after the competition training period (phase 3) but not after the more anaerobic intensive training period (phase 2).

A panel of cell activation markers and cell-adhesion markers were also measured by Baum et al. (28), as well as cell function. Intercellular adhesion molecule 1 (ICAM-1), which plays an important role in T cell activation (10), was assayed on monocytes. ICAM-1 is a membrane protein that is a specific ligand for lymphocyte function-associated antigen - 1 (LFA-1). ICAM-1 is present on cells expressing major histocompatability proteins, and LFA-1 is present on effector cells (T cells, B cells, granulocytes, and monocytes). The binding of ICAM-1 and LFA-1 is an important step in cell-cell interaction and effector cell activation (10). The number of monocytes containing ICAM-1 rose in athletes after each training period compared to the previous determination, but none of these differences were significant. There were significantly more ICAM-1 containing monocytes in athletes as compared to controls when the athletes finished the competitive training period (phase 3). The increased number of monocytes as training progressed, may help explain why the number of monocytes expressing ICAM-1 increased. Soluble ICAM-1 (s-ICAM-1), measured in blood, was significantly higher in the athletes after the endurance training period (phase 1) than for the controls. The authors suggested that this increased expression of ICAM-1 on monocytes may be indicative of activation of the monocytic-phagocytic system. Baum et al. (28) also suggested that an elevated soluble ICAM-1 concentration in blood is indicative of an enhanced protection against infection, as they cited a paper by Marlin et al. (36) that found that s-ICAM-1 inhibited rhinovirus infection.

The percentage of T_{helper} cells which had IL-2 receptors on them (called IL-2-R-positive helper cells) was significantly increased after the competitive training period (phase 3) compared to after the endurance training period (phase 1). Soluble IL-2-receptor concentration was greater in athletes compared to controls following all three training phases, but only significantly so when the athletes had just completed the competition training phase. IL-2 is a cytokine that is important in launching cell-mediated immune responses. It was originally called the "T cell growth factor". IL-2 is the major autocrine growth factor for T lymphocytes, and the quantity of IL-2 synthesis by activated T_{helper} cells is an important determinant of the magnitude of T cell-dependent immune responses (10). IL-2 also stimulates natural killer cell and B cell growth (10). Receptors for IL-2 are formed on lymphocyte membranes as the requirement for stimulation by IL-2 rises. Cell surface receptors are then shed when the receptor is not required by the cell, and soluble pieces of the receptors can be measured circulating in the blood. Increases in circulating soluble IL-2 receptor concentration is indicative of increased expression of IL-2 receptors on cells due to activation of cell-mediated immunity. Baum et al. (28) reported that mean athlete s-IL-2-R levels were greater than controls by a factor of 2. It was noted that some athletes had normal s-IL-2-R levels but others had s-IL-2-R levels in the ranges seen in active immunological disease. Baum et al. (28) suggested several possible reasons for such high s-IL-2-R levels in some but not all athletes: ① genetic differences, ② that some training adaptations are anti-infectious or stress-reducing and thus are responsible for normal values, ③ that athletes under intensive stress show high values of s-IL-2-R, ④ that high circulating concentrations of s-IL-2-R are part of an acute-phase-reaction or are related to physiological activation of the immune system, which is necessary for immunological function after exercise and a protective adaptation against non-specific infections, and ⑤ that attachment of IL-2 results in suppression of the immune system.

Baum et al. (28) reported that the proportion of certain lymphocytes that were activated changed over the different training phases. The proportion of B cells that were activated was significantly higher in athletes compared to controls after the endurance training phase (phase 1) and was greater, but not significantly so, after the increased anaerobic training phase (phase 2). The proportion of T_{helper} cells that were activated was greater in athletes compared to controls after all of the training phases, although results were not statistically significant.

The proportion of T_{helper} cells that were memory cells (CD45RO^{+} T_{helper}) increased significantly from after the endurance training phase (phase 1) to after the competition training phase (phase 3). Baum et al. (28) suggested that the increased proportion of memory T_{helper} cells was a result of the intensified physical and psychic stress during the competition phase (phase 3).

Finally, oxidative capacity of granulocytes was measured and it was found that the athletes had significantly lower values than did the controls, after endurance training (phase 1) and increased anaerobic training (phase 2) phases. Following the competition training period, granulocyte oxidative capacity was not significantly different from that of the controls. Baum et al. (28) did comment that they felt there were some errors in the methods used to determine these measurements, but they did not expand on this idea.

In summarizing the results from the study by Baum et al. (28), total monocyte count was significantly higher after the competition phase of training (phase 3) as compared to after endurance training (phase 1). There were no other significant changes in leukocyte or lymphocyte subset counts when the athlete's immune cell counts, obtained following each training phase, were compared. A weakness of this study is that there were no baseline values as no pre-training measurements were obtained. Several measurements of immune cell activation state were obtained. These activation measurements did suggest that the immune systems of athletes were and/or had been more active than that of the controls.

Baj et al. (29) conducted a longitudinal study where a panel of immune parameters were measured pre- and post- training in young (aged 21 ± 1.5 years) competitive cyclists. Fifteen male cyclists underwent a VO_{2max} test and blood collection in March, after a winter pause in training, and another in August, after 6 months of intensive training and racing. Average cycling mileage was 500 km per week. Sixteen healthy non-trained male students (aged 20.5 ± 2.1 years) served as the control group. The control group participated in both March and August tests.

Results from the pre-training and racing March tests indicated that there were no significant differences between cyclists and controls in leukocyte counts, neutrophils, total lymphocytes, T_{helper} ($CD4^+$) cells, $T_{cytolytic}$ ($CD8^+$) cells, $CD4^+/CD8^+$ ratio, and natural killer ($CD16^+$) cells. Total T ($CD3^+$) cells were significantly lower in athletes compared to the controls.

After 6 months of intensive training and racing, several significant changes in cell numbers were reported by Baj et al. (29). Compared to the control group, cyclists had significantly lower leukocyte and total lymphocyte counts. Total T ($CD3^+$) cells, T_{helper} ($CD4^+$) cells, and the $CD4^+/CD8^+$ ratio values for the cyclists were significantly diminished in August as compared to controls and as compared to their own values before training and racing (in March). Cyclists had significantly depressed lymphocyte IL-2 generation in post-training and racing (in August) as compared to their pre-training (March) values and as compared to August control values. Baj et al. (29) remarked that the significant decrease in the capacity of lymphocytes to generate IL-2 was probably due to the diminished T_{helper} ($CD4^+$) cell number, as it is known that $CD4^+$ cells are the main producers of IL-2.

Baj et al. (29) reported that cyclists had elevated lymphocyte proliferation by phytohemagglutinin-A (PHA) and anti-CD3 monoclonal antibodies, but not by concanavalin-A (Con-A) or pokeweed mitogen (PWM) when comparing post- (August) to pre-training and racing (March) values. Baj et al. (29) postulated that this increase in lymphocyte proliferation by PHA and anti-CD3$^+$ mAb observed simultaneously to reduced CD3$^+$ and CD4$^+$ cell numbers might be considered as a compensatory effect.

Granulocyte oxidative burst was measured using chemiluminescence with three separate stimuli (N-formyl-methionyl-leucyl-phenylalanine (*f*MLP), phorbol myristate acetate (PMA), and opsonized zymosan (OZ)) and in non-stimulated resting cells. Results showed that granulocyte oxidative burst was significantly decreased in athletes in August compared to in March in non-stimulated and in PMA and *f*MLP stimulated granulocytes. *f*MLP stimulated granulocyte oxidative burst capacity was significantly decreased in athletes as compared to controls in both March and August. Baj et al. (29) suggested that in August, granulocytes may have been "exhausted" due to their continuously activated effort in dealing with lesioned muscle cells that are the result of the acute effort required in intensive training and racing.

Baj et al. (29) concluded that the changes seen in immune function in this study are probably a result of "chronic exposition of the immune system to the influence of hormonal changes, hyperventilation or metabolic acidosis due to anaerobic effort, hyperthermia, dehydration, osmotic and plasma electrolyte balance". They stated that each of these factors increase the risk of infection irrespective of immune status. The authors felt that this study supported the idea that long-term intensive physical effort is harmful to immunity based on the fact that total T ($CD3^+$) cells were significantly diminished in number and that granulocyte oxidative burst and IL-2 generation were significantly diminished.

In summarizing the findings by Baj et al. (29), it was found that total T cell and T_{helper} cell counts and the $CD4^+/CD8^+$ ratio were significantly decreased in athletes after they had undergone a 6 month period of heavy endurance training and competing. A number of significant changes in immune cell functional measurements were also reported. Baj et al. (29) concluded by stating that long-term intensive physical effort had a harmful effect on immunity.

Wolfarth et al. (37) reported no statistically relevant changes in any of the cellular and humoral immune parameters measured in 16 regional and national top class athletes participating in cross-country skiing and road cycling over an 8-week training period. This reference was obtained from an abstract and therefore the precise methodology was not indicated. The general conclusion was that these top athletes did not show an increased susceptibility to infection, nor a significant change in immune parameters over the 8-week training period.

2.2.2.4 Immune Responses to Long Term (> 3 weeks) Moderate Endurance Training

It is important to review studies which have investigated immune responses to moderate training protocols in order to determine if the effects, or lack thereof, of this training on immune parameters can be differentiated from heavier training protocols. One such moderate training protocol longitudinal study was carried out by LaPerriere et al. (30). Fourteen healthy sedentary males, between the ages of 18 and 40 years, were randomly assigned to an aerobic training or control condition. The aerobic training consisted of three 45 minute sessions of cycle ergometry exercise per week at 70-80% of age predicted maximum heart rate for ten weeks. Blood samples were obtained pre- and post-training in order to measure total white blood cell count, percentages of total lymphocytes and monocytes, lymphocyte subset counts, and certain lymphocyte activation markers.

LaPerriere et al. (30) reported that there were no significant differences in total white blood cell count and percentage of lymphocytes and monocytes between exercisers and controls, and between pre- to post-training measurements in either group. There were, though, significantly elevated total T ($CD2^+$) cells, activated T ($CD2^+TA1^+$) cells, T_{helper} ($CD4^+$) cells, $T_{inducer}$ ($CD45RA^+CD4^+$) subset of T_{helper} cells, $T_{cytolytic}$ ($CD8^+$) cells, and B ($CD20^+$) cells in the exercise group post-training as compared to pre-training. No pre- to post-training changes were seen for the non-exercising control group. When comparing the post-training results of the exercise group to the post-training results of the control group, significant elevations in the lymphocyte subsets of $CD2^+$, $CD2^+TA1^+$, $CD4^+$, $CD45RA^+CD4^+$, and $CD8^+$ cells were seen.

LaPierriere et al. (30) suggested that a greater number of available circulating immune cells results in an improved immune response. They suggested that larger numbers of $CD4^+$ and $CD45RA^+CD4^+$ cells could result in more IL-2 production, which could activate more $CD8^+$ cells and thus cell-mediated immunity could be augmented.

Humoral immunity was suggested to have a potentially improved response because of the increased B cell (CD20$^+$) concentrations following training.

The researchers conducting this study were careful to obtain their blood samples at a consistent time in the morning and at least 48 hours after the last bout of exercise in order to eliminate any immunomodulatory effects of acute exercise. Subjects also presented themselves for blood collection after fasting overnight.

LaPierriere et al. (30) concluded that these results did indicate a potential beneficial increase in cellular and humoral immunity as a result of aerobic exercise training, although they questioned if the elevated lymphocyte numbers represented an enhanced recruitment of lymphocytes from lymphatic organs to the periphery due to the short length of time between the blood test and the last exercise session.

The three heavy training load studies (28, 29, 30) reported conflicting changes in resting leukocyte and lymphocyte count responses to heavy endurance training. Baum et al. (28) and Wolfarth et al. (37) reported no changes in immune cell counts, whereas Baj et al. (29) reported a marked drop in leukocyte and lymphocyte numbers. Both sets of authors reported significant alterations in immune cell activation and/or function measurements. Wolfarth et al. (37) concluded that eight weeks of heavy endurance training did not increase an athlete's susceptibility to infection. Baum et al. (28) suggested that the immune system was more active in heavily training endurance athletes as compared to non-exercising controls as a result of a potentially greater inflammatory response to exercise-induced muscle damage, and due to a greater ventilation rate which could place an athlete at a greater risk for upper respiratory tract infection. The authors did not report any sign of a depressed immune system, just a immune system that appeared more active than that of the controls, or when compared across the differing training phases. Baj et al. (29) did suggest that long-term heavy intensive endurance training did have a harmful effect on the capacity of an athlete's immune system to combat infection. Baj et al. (29) reported that immune cell counts were depressed, and that granulocyte oxidative function may have been strengthened in compensation for the decreased lymphocyte counts. LaPierriere et al. (30) reported they felt moderate exercise had an immune enhancing effect, as demonstrated by elevations in several lymphocyte subset counts and elevations in certain cell activity markers. The results of these studies suggest that heavy endurance training does cause alterations in the immune system. Baum et al. (28) reported on athletes with active but strong immune systems that are able to combat the added stress imposed by the training. The results from Baj et al. (29) suggested that their athletes may have approached a point at which their immune systems had become suppressed as a result of the increased stress imposed by training and racing. Determining the optimal methods with which to measure immune function will help sport scientists and coaches counsel athletes on optimal training phases.

2.3 Stress and Immunocompetence

It is apparent that acute exercise has an immunomodulatory effect. It seems as though chronic aerobic training has an immunomodulating effect, but the mechanism of this effect is not well understood. The remainder of this literature review is directed towards the possible mechanisms by which aerobic endurance training could have an effect on the immune system. Stress has been reported to affect immunocompetence

(38). The physiology of the non-specific stress response and humoral modulators of the stress-immunocompetence communication pathways will be reviewed, including cytokines and the hormone cortisol. Testosterone is an anabolic hormone that has been reported to be inhibited in stressful states (38) and has been a commonly measured factor in exercise studies measuring stress and an organism's ability to maintain a productive, healthy, functional physiological state. A short review of the effect of chronic training on blood testosterone concentrations will conclude this review of literature.

It is well known that stress and immunocompetence are inter-related (38). Experimental animal research studies have shown that laboratory stressors such as forced exercise, avoidance learning, restraint, isolation, and cold exposure make animals more susceptible to primary infection with a variety of viruses and bacteria (39). There are, in fact, centuries of clinical observations regarding individuals who became sick following stressful situations (40). It can be concluded from research on the effects of stress on pathogenesis and immunity during infection that there is evidence for an association between stress and the pathogenesis of infectious disease (41).

Heavy overload endurance training may be a stressor that is strong enough to cause alterations in immunocompetence. Anecdotal observations suggest that athletes undergoing heavy training or competition become ill at a greater rate than do individuals in the non-athlete population (1). Demonstrating the effects of exercise on the prevalence of infection, both rabbits (42) and guinea pigs (43) showed enhanced resistance to infection when regular exercise was conducted prior to infection by pneumococcus, whereas fatiguing exercise at the time of infection increased the animal's susceptibility.

2.3.1 The General Adaptation Syndrome

Selye (44, 45) suggested that any stressful stimulus would produce specific and non-specific stress responses in an organism. He called the physiological symptoms of the non-specific stress response the General Adaptation Syndrome (G.A.S.). The four stages of the G.A.S. were:

① the initial "alarm reaction", characterized by an immediate sympathoadrenomedullary discharge,

② the stage of resistance, characterized by activation of the hypothalamic-pituitary-adrenal (HPA) axis,

③ adrenal hypertrophy, resulting in an increase in glucocorticoid production and a thymic and lymphoid shrinkage, and,

④ deterioration and death, resulting from "diseases of adaptation", i.e. those brought on by the G.A.S. (46).

Typical hormonal manifestations of the G.A.S. were described as

① involution of the thymico-lymphatic apparatus;

② eosinopenia;

③ appearance of gastro-intestinal ulcers;

④ enlargement of the adrenal cortex, with discharge of its lipids and cholesterol;

and, ⑤ increased elimination of corticoids and corticoid metabolites (44).

The stages and symptoms of the G.A.S. indicate that both the neuroendocrine and immune systems are affected in the stress response. Neuroendocrine - immune interactions have been suggested to be the mediators of the effects of stress on infectious disease (41).

2.3.2 Effects of the Non-Specific G.A.S. Stress Response on the Immune System

The non-specific G.A.S. stress response is the name for the physiological changes that are loosely called the "fight-or-flight" response. During a stress response, following an initial sympathetic charge, the adrenal cortex actively secretes mineralocorticoids and glucocorticoids (47). Mineralocorticoids promote the retention of sodium and water by the kidneys, thus increasing blood volume and blood pressure (47). Glucocorticoids maintain gluconeogenesis from protein, facilitate fat metabolism, support vascular responsiveness, modulate central nervous system function, and suppress immune function (48). It is the elevated adrenal secretion of glucocorticoids that may be the key connection between stress and immunomodulation.

Glucocorticoids have been shown to have a wide range of effects on the immune and inflammatory responses in animals and man (49). Glucocorticoids have been shown to inhibit the production of many different cytokines, including IL-1β, IL-6, TNFα (50). Glucocorticoid receptors have been found on several lymphocyte cell surface membranes (51) and glucocorticoids have been shown to kill lymphocytes by apoptosis (52). Glucocorticoids are potent immunosuppressive agents and have been used for many years in clinical practice to control allergies, inflammation, and autoimmune disease

(53). There have been several studies that have shown that cortisol production is raised in psychologically stressful conditions (54, 55, 56, 57) and that psychologically stressful conditions have immunomodulatory effects (58, 59, 60). Since it is well known that corticosteroids have immunomodulatory effects and that stressful conditions cause elevations in glucocorticoid secretion, it is plausible that the production of cortisol during stressful conditions plays a role in immune suppression.

Glucocorticoids have been reported to be potent suppressors of reproductive function (38, 61), and reproductive function has been reported to be suppressed during stressful periods (38). Endurance athletes have been reported to have reduced circulating testosterone concentrations when compared to sedentary controls (62) and after an increased training load (63).

The responses of circulating glucocorticoid and testosterone concentrations in chronically endurance training individuals will now be reviewed. First though, a short review of how certain cytokines play a role in modulating neuroendocrine - immune interactions is included, in order to explain the reasoning for including the measurements of serum IL-1β, IL-6, and TNFα in this study.

2.4 The Cytokine Connection

IL-1β, IL-6, and TNFα are all classified as mediators of natural immunity and are considered to be produced by and secreted mainly from mononuclear phagocytes (10). The functions of these cytokines are many. For an overview of the function of these cytokines and others, please refer to Appendix C.

2.4.1 Cytokines as Chemical Mediators between the Neural, Endocrine, and Immune Systems.

Psychoneuroendoimmunology is a new and growing field. It is the study of bi-directional communication pathways between the neural, endocrine, and immune systems. Cytokines act as primary messenger molecules between these systems. Cytokines do not act exclusively within the confines of the immune system. The physiological response to the stress of exercise and the possible immunomodulating effects are examples of neuroendocrine - immune interaction.

The hypothalamo-pituitary-adrenocortical (HPA) axis produces glucocorticoids that suppress the immune system in order to protect the organism from the pathophysiological responses of a continually over-active immune system (64). For example, it is thought that certain autoimmune diseases develop in an organism as the result of defects in HPA-immune communication (65). Activation of the HPA axis impedes excessive immune reactions and therefore halts deleterious autoaggressive (autoimmune disease) (65). Cytokines play an important role in the communication between the HPA axis and the immune system. Please see Figure 2-1 for an overview of these communication pathways.

IL-1β (64), IL-6, TNFα all activate the HPA axis (66). An activated HPA axis secretes more glucocorticoids, which feedback to potently suppress cytokine production (65). Glucocorticoids feedback to suppress their own production by acting to suppress the production of corticosteroid releasing hormone (CRH) and adrenocorticotropic hormone (ACTH).

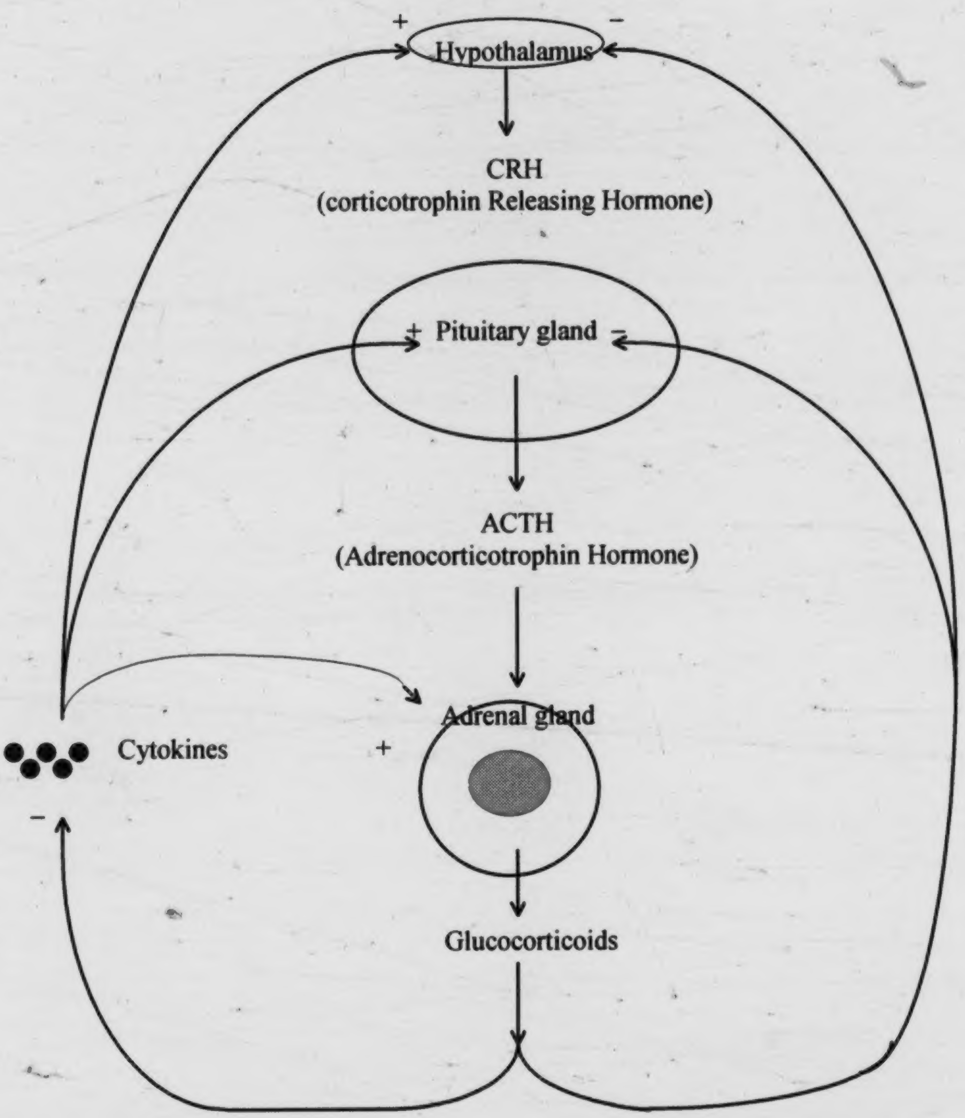
+
−
Hypothalamus
CRH
(corticotrophin Releasing Hormone)
+ Pituitary gland −
ACTH
(Adrenocorticotrophin Hormone)
Adrenal gland
Cytokines
+
−
Glucocorticoids

Figure 2-1. Communication pathways between the neuroendocrine and immune systems. The information for this figure was obtained from figure 2 of Imura, H., Fukata, J., and T. Mori (1991) Cytokines and endocrine function: an interaction between the immune and neuroendocrine systems. **Clinical Endocrinology** 35: 107-115.

It is difficult to predict how the concentrations of IL-1β, IL-6, and TNFα react to the stress of chronic endurance exercise. They could be elevated in order to promote glucocorticoid secretion, or they could be suppressed as a result of glucocorticoid secretion. Additionally, the question arises as to how the cytokine response to chronic attacks endurance exercise would be affected by an immunological challenge that requires cytokines to play a role in inflammation and/or an immune response. An immunological challenge could alter cytokine concentrations for reasons that are due to immunological challenge and thus are unrelated to those due to chronic endurance training.

2.4.2 Cytokines as Mediators of the Acute Phase Response.

The response of cytokines to muscle damage is another means by which blood cytokine concentrations may be affected by exercise. Muscle protein damage resulting from excessive exercise and/or the lack of recovery, as may be found in heavy overload endurance training (67), causes the immune system to be activated in order to rid the disrupted site of damaged muscle tissue (68). Inflammation and pain are normal signs of immune system reactivity. Circulating IL-1β, IL-6, and TNFα have been reported to be elevated after acute strenuous exercise (68). The acute phase response is activated after such intense exercise sessions. IL-1β, IL-6, and TNFα all play important roles in the acute phase response (10). The purpose of the acute phase response is to increase resistance to infection by altering metabolism to promote wound repair and by activating both cellular and natural immune reactivity (69). The acute phase response is characterized by fever and an increase in granulocyte production (70). Acute phase proteins, such as fibrinogen, haptoglobin, and c-reactive protein, are synthesized and released; and endocrinological changes, such as the increased production of ACTH, cortisol, and adrenal catecholamines occurs (70). There are remarkable similarities between the acute-phase response and the physiological response to exercise, including a significant increase in IL-1 activity immediately and 24 hours after exercise. This increase in IL-1 activity may be pivotal in linking the haematological and immunological changes observed after sustained strenuous exercise (69). In addition to elevated circulating IL-1β concentrations, TNFα and IL-6 have been reported to rise during and following acute physical exercise (71). Since cytokines are molecules which affect immune function, the alteration in circulating cytokine concentrations during an acute phase response brought about by heavy exercise may have an effect on the immune function of an athlete who trains heavily regularly.

There are other moderators of the HPA axis. Peptides, such as α-, β-, and γ-endorphins have been reported to counteract the activating effects of IL-1 and other cytokines on the HPA axis (72), thus, they may act to inhibit HPA axis activation.

The immune and neuroendocrine systems are complicated. Knowledge in this area is still fairly new and many researchers are now beginning to study in this field. The study of the effect of exercise on these systems is popular at this moment due to advances in biotechnology which allows researchers to study biochemical variables easily and relatively inexpensively. Large gains in knowledge are being achieved. However, how circulating cytokine concentrations respond to heavy endurance training is not well understood and is difficult to predict because of the complex roles of cytokines.

2.5 Cortisol Responses to Endurance Exercise

2.5.1 Responses of Circulating Blood Cortisol to Acute Exercise

The response of the production of the glucocorticoid cortisol to an acute exercise load is related to intensity of exercise load. Luger et al. (73) demonstrated that plasma cortisol concentration showed intensity-dependent elevations in men running on a treadmill for 20 minutes. Running at 50% of their $\dot{V}O_{2max}$ elicited no increase in plasma cortisol concentrations. Running at 70 and 90% of $\dot{V}O_{2max}$ elicited significantly elevated plasma cortisol concentrations. Larger increases were seen at 90% of $\dot{V}O_{2max}$ than at 70%). There was a short (minutes) delay in the rise in circulating cortisol concentrations following the start of an exercise effort. The more stressful the exercise, the sooner the increase in cortisol production was seen. The fact that there was a delay in cortisol rise, i.e. a delay until the body found the exercise stressful, was demonstrated in several other studies (74, 75) in addition to Luger et al. (73). It has been accepted that cortisol production is elevated in response to exercise, showing increasingly larger elevations as the strenuousness of the exercise becomes greater.

2.5.2 Response of Cortisol to Endurance Training

If acute strenuous exercise causes an elevated adrenal production of cortisol, it is plausible that many exercise bouts with little recovery between them, such as in training, could cause hypercortisolemia. This has been shown to occur in some studies (73, 76, 77, 78, 79, 80, 81) but not in others (82, 83, 84, 85, 86, 87).

2.5.2.1 Elevated Resting Cortisol Responses to Heavy Endurance Training

Roberts et al. (77) reported that resting plasma cortisol concentrations were elevated following a 2 week period of overtraining when compared to baseline values. Five males "overtrained" by doubling their mileage and maintaining a constant intensity. Endurance training modes were either running, swimming, or cycling. Frequency of training was greater than 4 days per week. Subjects were considered overtrained when their resting heart rate was elevated and when they demonstrated poor recovery time. These criteria were supported by the occurrence of a diminished $\dot{V}O_{2max}$ value. The subjects performed the two weeks of doubled mileage training three times (interspersed with 6 - 8 weeks of regular training) before experiencing symptoms of overtraining. The statistical power of this study is fairly low with five subjects; therefore, fairly large changes in plasma cortisol concentrations were required in order to obtain significant changes. Only one sample of plasma cortisol was obtained as a pre-overtraining measurement rather than a series of measurements taken in order to take the average. Hormones, such as cortisol, are released in pulses and are found in varying concentrations in the blood depending on sampling time. For such a variable measurement, it would have been better to take numerous samples and average the results. The protocol for blood collection was standardized in a very strict manner, though, by following strict guidelines. Subjects reported to the laboratory at 7:00 a.m. after having fasted for 8 hours and rested quietly in the supine position for a full hour with a heparinized catheter inserted into a forearm vein before samples were drawn.

Kirwan et al. (78) reported that the elevation of resting serum cortisol concentrations appears to be a normal response to the stress of an increased training load. Twelve highly trained male collegiate swimmers completed 10 days of training that was designed to impose a sudden increase in training load. Training mileage doubled from an average of 4,266 (± 264) $m{\cdot}d^{-1}$ to 8,970 (± 161) $m{\cdot}d^{-1}$, while intensity was maintained at ~95% of $\dot{V}O_{2max}$. Kirwan et al. (78) specifically mentioned that no decrease in performance was seen, so these subjects could not be classified as overtrained. Kirwan et al. (78) did report that subjects found the training very difficult, as shown by increased ratings of perceived effort and by the coach's subjective assessment, thus subjects did experience an increased psychological stress along with the increased physical training stress.

Neary et al. (76) reported that cortisol concentrations, measured in urine pooled and collected for a period of 24 hours, rose in cyclists during and after heavy endurance training when compared to pre-training values. Twenty-seven male cyclists participated in a training program that lasted seven weeks. The training program for the initial 2 weeks consisted of cycling at 75% of $\dot{V}O_{2max}$ for 45 minutes per day, four days per week. The training program for the subsequent five weeks consisted of cycling at 80 to 85% of $\dot{V}O_{2max}$ for 60 minutes, five days per week. Urinary free cortisol was significantly higher than pre-training values following weeks three through five, and was significantly elevated after week seven as compared to all of the previous weeks.

Seidman et al. (79) concluded that hypercortisolism may be present in subjects undergoing long term (~18 weeks) strenuous physical activity. Mean resting serum cortisol concentration was reported to have risen significantly after the 18 week study period when compared to pre-training values. The training regimen consisted of "aerobic exercise training" and prolonged training marches with back packs weighing up to 20 kg. Training was progressive, with weekly increases in load. Caloric intake was kept constant and at a fairly low level throughout the 18 week period. Mean caloric intake was 1997 ±772 kcal/day after six weeks and 2036 ±741 kcal/day after 12 weeks. Mean energy expenditure was 1934 kcal/training day during the first six weeks and 4100 kcal/training day after 12 weeks. These values do not include basal energy expenditure or leisure time energy expenditure. Thus, it is apparent that the subjects were under stress due to a lack of energy intake for the amount of energy expenditure. Seidman et al. (79) did not mention how much rest subjects received, other than one night's sleep, before blood samples were taken. It is interesting that resting serum cortisol concentrations were not elevated at 6 and 12 weeks of training compared to pre-training values, even though training load was progressively increasing. Perhaps the training was not yet strenuous enough to cause the hypercortisolemic response that was seen at 18 weeks

Kraemer et al. (80) studied the effects of three different run training programs on the responses of cortisol to maximal exercise and pre-exercise cortisol. The three run training programs were each 10 weeks in length and could be generally described as sprint interval training (SI), endurance (E), and combined sprint interval and endurance (C) training. The C training group did all of the exercise training sessions of both the SI and E groups combined. Resting plasma cortisol concentrations rose significantly in the

SI and C groups, as compared to pre-training values, but not in the E group. The SI and E training regimens were not as strenuous as the C group. The SI group trained 5 days per week. Each of their training sessions consisted of two sets of four 20-second maximal interval run sprints. 1 minute of rest was given between each 20-second interval in a set and 5 minutes was given between the two sets. The E group trained 3 days per week. In each training session, subjects ran as far as they could in 30 minutes. Mean exercise intensity at 15 minutes into the run, determined by heart rate, was 80.0 ± 1.9% of their treadmill $\dot{V}O_{2max}$. The C group trained 6 days per week and did both SI and E's training programs. $\dot{V}O_{2max}$ results for all groups improved significantly at 8 weeks compared to pre-training values, and stayed elevated at 10 weeks. The authors gave no indication that they felt the subjects were overtraining. They did feel that the high lactate levels induced by the interval training may have had a possible anaerobic influence upon the release of ACTH and cortisol.

Fry et al. (81) reported no significant changes in serum cortisol concentrations in fifteen elite national and international athletes when all were training properly and in good health, but when three of the athletes became overtrained, two of them had elevated resting serum cortisol concentrations. Overtraining was defined in the discussion as a defined loss of performance when the athlete was in a state of recovery, so that the expectation of an optimal performance was realistic. The subjects in this study were all elite national and international athletes: 3 middle distance runners (all male) and 12 rowers (4 women and 8 men). All subjects were monitored while they carried out their own training programs. Tests were carried out over a 6 month period, over which the athletes trained leading up to their major competitive events. Tests were held every 6 weeks. The researchers were careful to ensure that during the 3 days leading up to each test, training intensity and volume was low although it was not mentioned how many hours of no exercise subjects had before tests. The paper did infer that training was held on the day preceeding the testing day. The time of day that blood samples were collected was described as being during the "afternoon". Considering that hormones such as cortisol are released pulsatilely and are therefore represented in the blood stream in oscillating concentrations, cortisol measurements obtained at varying times during a day increases the potential for error in the methods of the study.

These studies all support the hypothesis that stressful exercise training, to the point of overtraining in some cases, causes resting hypercortisolism. Reading these papers does raise some questions about this hypothesis. Kirwan et al. (78) distinctly state that a rise in resting cortisol is a normal response to the stress of an increased training load. This idea is different from an altered homeostatic or base hypercortisolemia. Short term hypercortisolemia is all that Kirwan et al. (78) could conclude considering their study's training program was only 10 days in length, but this idea of hypercortisolemia during a period of adaptation to an increased training load is supported by Neary et al. (76). In the study by Neary et al. (76), increases in resting 24 h urinary cortisol mirrored the increases in training load throughout the 7 weeks. Training load rose during the first week and during the third week. Cortisol rose (not significantly) in the first week of training and then plateaued in the second week of training. Cortisol then rose again following week three (not a significant change from week two) when the training load was increased again. It then plateaued while the athletes continued training at this load. By week four, urinary free cortisol was significantly higher than pre-training values. During the final week of training, the authors mentioned that all of the training was performed at greater than 85% of $\dot{V}O_{2max}$, which was a higher intensity than that trained at in the previous weeks, and cortisol concentrations rose again, significantly this time compared to week six. Thus, it does seem as though resting cortisol concentrations rise when the organism is in an adaptation mode. When homeostasis is approached, cortisol concentrations level out.

The idea that cortisol production is raised when athletes are in the physiological state of adaptation is supported by the study of Fry et al. (81), in that two of the three athletes considered to be overtrained had sustained, significantly elevated cortisol concentrations. Overtraining is a condition where performance suffers as a result of the athlete being unable to recover optimally from the training load imposed. Since "recovery" is the active, adaptive physiological process the body is carrying out to return to homeostatic norm, or a supercompensated state after an overload stimulus, overtraining could be considered a condition of continual attempted recovery.

Even though there seems to be a convincing hypothesis that hypercortisolemia occurs in the heavily training and overtraining athlete, an argument well backed up by numerous papers, there are several studies that do not support this hypothesis. A short review of several of these studies will now follow.

2.5.2.2 Depressed Resting Cortisol Responses to Endurance Training

A study by Lehmann et al. (82) in which the authors attempted (and succeeded) in inducing an overtraining syndrome in 8 experienced middle- and long-distance runners demonstrated that 24 hour urinary cortisol excretion fell to 70% of baseline values. The training program was broken down into 4 one-week periods. Running distance was increased individually each week by about 33%. Mean running distance for week one was 85.9 km; for week two was 115 km; for week three was 143.1 km; and for week four was 174.6 km. 90% (wk 1) to 98% (wk 4) of the training was at an intensity between 50 and 70% of maximum performance. From 10% (wk 1) to 2% (wk 4) of the training was spent in interval and high speed runs. Some researchers have classified overtraining into two types: sympathetic and parasympathetic (82). Sympathetic overtraining, characterized by elevated cortisol production, has been reported to be seen at the onset of overtraining. Parasympathetic overtraining is characterized by suppressed levels of resting cortisol (82). Lehmann et al. (82) suggested that a parasympathetic overexertion syndrome may occur more frequently with high volume endurance training. The results of Lehmann et al. (82) support the idea that excessive high volume endurance training causes parasympathetic overtraining and a subsequent fall in resting blood cortisol levels.

Dressendorfer and Wade (83) reported that plasma cortisol concentrations were not significantly different one day following a 15 day endurance running race when compared to one or two days before the race. Nineteen experienced male endurance runners competed in a 400 km road race. They commenced each day, of the fifteen days, at 7:00 a.m. and ran distances ranging from 15 to 34 km, and averaging 26.7 km·d^{-1}. About 45% of the course included rolling to steep hills. The runners maintained their usual training speeds even though the race distance was twice their regular mileage. Blood was collected 20 - 26 hours after exercise, both prior to and following the 15 day race, at the same time of day (± 1 hour) for each subject. Samples were drawn 8 - 10 hours after eating and after the subject had rested in the seated position for 10 - 15 minutes.

Vervoorn et al. (83) reported that in elite rowers, over the course of 35 weeks of heavy training, resting serum cortisol values did not significantly change. Cortisol levels appeared to mirror, in the opposite direction, training volume. In other words, as training volume rose, resting serum cortisol concentration fell. The fall in cortisol was not significant though. Although the results of this study failed to demonstrate a significant change in cortisol, small changes in reverse to training stress were apparent.

Several other studies have reported no significant changes in resting cortisol concentrations in endurance athletes undergoing heavy training. Flynn et al. (85) reported that no changes in resting serum cortisol occurred in 8 male collegiate cross-country runners or in five male collegiate swimmers on four strategically placed blood samples drawn throughout their competitive seasons. Sample collections were performed before and after periods of increased training load as well as after taper or eased training loads, in order to measure periods of varying training stress. Blood samples were taken soon after 7:00 a.m. following an overnight fast, and after resting in the supine position for 15 minutes. Flynn et al. (85) concluded that the usefulness of resting cortisol as a marker of training stress appeared doubtful.

Lopez-Calbet et al. (86) reported that basal salivary cortisol did not change significantly over a 6 month period during which seven elite male cyclists trained and competed in races. Seven male cyclists underwent two assessments, one at the onset of the competitive season in February, and the other 6 months later, in August, at the end of the competitive season. It was not mentioned if the assessment in August was soon after competition or if it occurred after a period of rest greater than a couple of days. These cyclists were amateurs, except for one professional. They all competed at the national level in Spain. Weekly riding distances ranged between 300 and 750 km. Two races, between the distances of 100 and 150 km, were usually completed on the weekends. In addition to the weekend races, they competed in five to eight stage races that lasted from 3 to 7 days each. Subjects were asked to lessen their activities for 48 hours prior to the hormone assessment. Salivary cortisol samples were collected under fasting conditions (it was not mentioned how long fasting occurred for), after 30 minutes of rest, and at 9:00 a.m. One sample was obtained at each February and August collection. Lobez-Calbet et al. (86) expected cortisol to rise and went as far as to say that salivary cortisol showed a non-significant tendency to increase.

Hooper et al. (87) reported that plasma cortisol concentrations did not vary significantly during the season for 14 elite, international level swimmers, and also were not significantly different throughout the season between the stale (overtrained) and well-trained (not overtrained) swimmers. The identification of overtraining was determined by monitoring performance and ratings of fatigue. If performance in the maximal effort swim failed to improve from early to late season, and high, prolonged ratings of fatigue were reported in the daily log books, an athlete would be considered overtrained or "stale". Hooper et al. (87) made sure that "staleness" was not in fact due to illness by measuring for normal leukocyte counts and normal erythrocyte sedimentation rates. Subjects trained according to the program set by his/her coach. Testing of the subjects occurred five times during the season at times that, as closely as possible, matched the following descriptions:

(1) early-season (while aerobic base training - 2 to 3 weeks after the start of training).

(2) mid-season (during intense training - 12 - 14 weeks after the start of training)

(3) late-season (during intense training - 5 - 6 weeks before national team trials for the world championships)

(4) during tapering (3 to 5 days before commencement of trials)

(5) post competition (1 to 3 days after the end of the trials, before training resumed)

2.5.2.3 Summary of Cortisol Responses to Endurance Training

In summarizing these studies that have investigated serum cortisol concentration responses to endurance training, the question of whether resting concentrations of cortisol are elevated in athletes as compared to non-athletes, or in heavily training athletes compared their pre-training values, has not been conclusively determined. There seems to be a general suggestion that hypercortisolemia is prevalent in heavily training individuals, but the results of published studies have not necessarily supported this idea.

2.6 Responses of Circulating Testosterone to Heavy Endurance Exercise

2.6.1 Response of Circulating Testosterone to Acute Exercise

Acute aerobic exercise caused a significant rise in circulating blood testosterone concentrations (88). The magnitude of elevation was proportional to exercise intensity and volume of muscle mass recruited (89, 90). Depressed circulating concentrations of testosterone have been reported during exercise lasting longer than three hours (90), and during recovery from exercise (89, 90). An explanation for the rise in testosterone concentration has been suggested to be decreased hepatic clearance (89, 90).

2.6.2 Responses of Circulating Testosterone Concentrations to Chronic Endurance Training

Male endurance athletes have often been reported to have reduced resting circulating testosterone concentrations (89, 90). A proposed mechanism underlying this decrease in circulating testosterone concentrations is that elevations in circulating adrenal hormones, such as corticotrophin (ACTH) and cortisol, have suppressive effects on testicular hormones (38, 90).

A survey of recent longitudinal endurance training studies that have imposed heavy training loads reveals inconclusive results regarding circulating testosterone concentrations. Testosterone has been reported to be depressed as the result of heavy training (63, 77, 83, 85), or not changed (76, 79, 81, 86).

2.6.2.1 Depressed Circulating Testosterone Concentrations

A cross-sectional study performed by Hackney et al. (91) compared four hour resting free and total testosterone concentrations between highly trained endurance runners (10 km time = 31:58, standard error (SE) 00:30; marathon time = 2:37:24, SE 00:12:00) and untrained males. Both total and free testosterone concentrations were lower in the endurance trained group as compared to the untrained group. Serum cortisol concentrations were not different between the trained and untrained groups.

A longitudinal study by Roberts et al. (77), which was described earlier, on page 46, reported that basal testosterone levels decreased as a result of three months of doubled training volume. This decrease in circulating testosterone concentrations was accompanied by an increase in circulating cortisol concentrations.

Dressendorfer and Wade (83) reported that plasma testosterone concentrations decreased significantly by 31% when experienced male endurance runners raced in a staged 400 km road race that lasted 15 days. This study's design was described earlier, on page 52. Cortisol concentrations remained unchanged.

Flynn et al. (85) reported that serum total and free testosterone were both significantly reduced when endurance swim training was substantially increased for a 2 week period. Furthermore, total and free testosterone increased following a reduction in training volume. Cortisol was not significantly changed during the increase or decrease in training volume.

Urhausen et al. (92) monitored serum free testosterone concentrations during a 7 week competition period in elite rowers. Free testosterone concentrations fell continuously and significantly over the course of the 7 weeks. Two rowers who stopped intensive training and competing at two and three weeks into the study, respectively, still participated in the blood hormone measurements. Their testosterone values did not fall, as did those for the subjects who were still training. Serum cortisol values did not change significantly over the seven week period.

Wheeler et al. (63) monitored testosterone levels in sedentary subjects who commenced a six month running regimen. Total and free testosterone concentrations decreased significantly from pre- to post-training. Cortisol concentrations also decreased.

2.6.2.2 Unaltered Circulating Testosterone Concentrations

Neary et al. (76), whose paper is described earlier on page 47, reported that serum testosterone concentrations were initially at the low end of the normal range and did not change significantly with training. Urinary 24 hour cortisol levels did rise significantly during training. Seidman et al. (79), whose paper is described earlier on page 47, reported that mean serum total testosterone levels increased significantly over the first six weeks of training and then decreased significantly over the second six weeks of training. At 18 weeks of training, they did not differ from pre-training levels. Thus, there was no overall change in pre- to post-training total testosterone concentrations. Serum cortisol did increase significantly. Lopez-Calbet et al. (86), whose paper is described earlier on page 53, reported no significant change in salivary testosterone concentration from pre- to post-training. The authors did report that testosterone concentrations showed a tendency to decrease, but the change was not significant. Cortisol concentrations were reported to show a non-significant tendency to increase. Fry et al. (81), whose paper is described earlier on page 49, reported no significant changes in the resting blood free testosterone concentrations of elite rowers and runners when measured at the end of four distinct six-week intensive-training cycles. Three of the fifteen subjects showed depressed performance capacities and were thus considered overtrained. Of these three overtrained subjects, one had elevated free testosterone levels, and two showed no significant changes in testosterone levels when compared to their own values when not in an overtrained state. The elevated serum free testosterone level occurred in conjunction with an elevated serum cortisol concentration. One of the other two overtrained subjects showed elevated serum cortisol concentrations, but no change in free testosterone concentrations.

Resting blood testosterone concentrations have been proposed to fall in response to heavy endurance training stress (89, 90), but the evidence from the exercise literature does not fully support this hypothesis (76, 79, 81).

The hypothesis that circulating cortisol concentrations rise and testosterone concentrations fall as a result of increased endurance training stress has been supported by some studies (77, 79, 86) but has also been weakened by others (63, 76, 81, 83, 85). Further research will be required in order to understand how these variables are affected by exercise training, as no final conclusions can be made at this time.

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Chapter 3

Immune Status in Male Cyclists during Heavy Endurance Training

3.1 Introduction

Recently it has been proposed that endurance training may suppress immune function and increase susceptibility to infection (1, 2, 3, 4, 5). However, the physiological mechanisms of exercise-related immunosuppression remain unclear. Immunological markers such as leukocyte and lymphocyte subset counts have been monitored over short (10 to 21 day) overload training periods (6, 7, 8, 9) and over longer (4 week to 6 month) periods (10, 11, 12, 13). Immune data have been compared in athletes vs. non-athletes (14, 15) and in overtrained athletes vs. normal values and/or controls (16). The findings of these investigations are inconclusive, as some studies indicated that alterations in immune cell counts occurred along with elevated training stress (7, 9, 10, 11, 12, 15, 17) while others did not (8, 13, 14, 16).

Acute strenuous endurance exercise has been reported to cause elevations in circulating concentrations of the cytokines IL-6 (18, 19, 20), IL-1β (21), and TNFα (22, 23). IL-6 has been shown to be elevated in rats in response to both physical and psychological stressors (24). There are few training studies that have monitored circulating concentrations of cytokines (12, 25). One longitudinal study reported that plasma TNFα rose during a combined endurance and strength training program in female but not in male subjects (25). However, TNFα and IL-1β have also been to be non-detectable during 4 weeks of heavy endurance training (12). IL-1β, TNFα, and interleukin-6 (IL-6) function in mediating the host inflammatory response in natural immunity (26). These cytokines also serve as important communication molecules between the immune and the neuroendocrine systems (27). Consequently, monitoring cytokine responses during training periods may indicate the presence of an immune response or of an inherent level of stress.

Resting serum cortisol and testosterone levels have been used to monitor the physiological stress of training. The typically expected responses are that cortisol should be elevated and testosterone depressed in stressed individuals (28). However, the results of other research have not always supported this hypothesis. Recently, Hooper and Mackinnon (29) suggested that changes in these hormone concentration are not necessarily reflections of overtraining. This supports the conflicting reports regarding the responses of these markers to training stress. Elevated resting cortisol (30, 31, 32, 33, 34, 35, 36) and depressed resting testosterone levels (34, 37, 38, 39) have been reported in conjunction with an increased training stress, while no changes in cortisol (37, 40, 41, 42, 43) and testosterone (6, 35, 43, 44) have also been reported. In fact, Lehmann et al. (45) reported depressed urinary cortisol concentrations in athletes who increased their mileage in an attempt to induce an overtraining syndrome. These conflicting findings may be partly explained by differences in the type, intensity, and duration of training, as well as the fitness level of the subjects. Furthermore, diurnal variability in blood hormone concentrations, coupled with the potentially dramatic effects of acute exercise, may further explain the inconsistent findings. Since there is a fairly compelling theoretical basis for their use, with careful control these variables may be viable markers of training stress.

The main purpose of this study was to monitor lymphocyte subset counts and selected cytokine concentrations over nine weeks of endurance training. The first six weeks emphasized continuous training at selected intensities around the ventilatory threshold. The next 18 days consisted mainly of interval training at intensities near $\dot{V}O_{2max}$. The final 10 days consisted of reduced volume cycling (unloading). Cortisol and testosterone were used as hormonal markers of training stress. The hypothesis was that the stress of overload training for nine weeks would perturb the immune system, demonstrated by chronic changes in lymphocyte subset counts and serum IL-1β, IL-6, and TNFα concentrations. Furthermore, it was hypothesized that training stress would result in elevated 24 hour urinary free cortisol and depressed serum testosterone concentrations. Finally, reduced training stress during an unloading period should allow the immune and hormonal values to return to baseline values.

3.2 METHODS

3.2.1 Subjects

Twelve healthy men volunteered to participate as subjects. Nine of these fully complied with the experimental protocol and represented the study sample. One subject dropped out of the study following the first week of training, and two subjects each missed approximately one week of training, which excluded their results from being utilized. All nine subjects were competitive mountain bikers. Institutional ethics review board approval for use of human subjects in research was obtained. Subjects signed informed consent documents that fully disclosed all study procedures and associated risks and benefits. Mean (±SD) characteristics of nine subjects at baseline were: age 24.7 ± 2.1 years, height 1.80 ± .03 m, body mass 76.0 ± 7.0 kg, maximal oxygen consumption ($\dot{V}O_{2max}$) 59.3 ± 5.0 $ml \cdot min^{-1} \cdot kg^{-1}$.

3.2.2 Design

The study followed was a quasi-experimental time series design (46). Each athlete acted as his own control during a four week baseline period. There was no separate control group since athletes will typically not allow themselves to be randomly assigned to a control group of no training. The lack of a distinct control group may decrease internal validity. Effects of history, maturation, and environment cannot be completely accounted for without a control group. However, it has been shown that were are greater between-subject variations over time than within-subject variations in lymphocyte subset counts (47, 48). This fact supports the choice of a time series design for this study, as subject's lymphocyte data following intervention were compared to their own baseline values, not to values obtained from control subjects. This supports the validity of utilizing a time series research design for monitoring lymphocyte subset counts over time. Additionally, the values of the dependent variables were stable over the baseline period. No statistically significant changes were seen in any variable. This stability in baseline measurements supported the validity of comparing post-treatment values to baseline values. Furthermore, such a design has frequently been favored for studying performance changes in athletes (49, 50).

The study duration was 15 consecutive weeks. This period included 4 weeks of baseline (B), 6 weeks of volume (V) training, 18 days of high intensity (I) training, and 10 days of unloading (U). See Figure 3 - 1 for a temporal organization of the study.

Whole blood leukocyte and lymphocyte subset count samples. Blood samples were obtained twice during B, twice during V and I, and once during U. The two B samples were obtained on consecutive days at the end of the phase. The mean of these two samples was used as the baseline measurement. For V and I, the first of two samples (V1 and I1) were taken during the final week of training, and the second samples (V2 and I2) were taken one week later during the testing period. The samples for U were obtained on the final day of the phase.

Serum cytokine and testosterone concentration samples. Blood samples were obtained at the same times that leukocyte and lymphocyte subset counts samples were drawn. However, one additional sample was taken at the beginning of the B phase. The mean of the second and third baseline samples was used as the baseline value other values were compared against.

Urinary free cortisol concentration samples. Urine was collected for 24 hours on the same days that the cytokine and testosterone serum samples were obtained. In all cases, blood samples were taken 36 - 44 hours after exercise, and the urine collection commenced 24 - 26 hours after exercise. This sampling protocol was chosen to allow adequate recovery time from the last exercise. A period of 24 hours was felt to be adequate time to rule out the acute alterations of an exercise effort. Immune cell counts have been shown to return to baseline values within 21 hours following an acute endurance exercise session (51). Alterations in percentages of circulating lymphocytes have been reported to return to normal after 24 hours (52, 53). Serum cortisol and testosterone concentrations were reported to have returned to baseline values by 2 hours following an anaerobic interval running session consisting of 25 - 1 minute intervals (54). Similarly, serum cortisol concentrations had returned to baseline values within 1 hour following a 2 hour endurance cycle ride at an intensity of 55 - 60 % of $\dot{V}O_{2max}$ (55).

3.2.3 Training Program

The purpose of the baseline phase was to establish consistent pre-training fitness values. During baseline, subjects were instructed to maintain their usual off-season training, without a change in volume, duration, or intensity.

All training during the V, I, and U phases was directly supervised. Training during the V phase consisted of five endurance and one strength work out per week, each on a separate day. Four of the endurance sessions involved continuous cycling on rollers or windtrainers at target heart rates ranging from 15 bpm below ventilatory threshold (T_{vent}) to 5 bpm above T_{vent}. T_{vent} was identified as the heart rate just below a distinct increase in $\dot{V}_E / \dot{V}CO_2$ during the third $\dot{V}O_{2max}$ performance test of the B phase. Please refer to Appendix B for further description on the determination of T_{vent}. Training heart rates are displayed in Table 3 - 1.

Exercise session duration was 50 minutes during the first week and increased by 5 minutes per week, to a maximum of 75 minutes. One session per week was continuous cycling at an intensity of 20 to 25 bpm below T_{vent}, starting at 90 minutes and extending to 180 minutes by the final week.

The I phase consisted of four high-intensity interval training sessions per week on Monark cycle ergometers. Intensity during the work intervals equaled the power output at which $\dot{V}O_{2max}$ was attained following the V phase. Interval duration progressed from eight 2- minute work intervals to a combination of six 2- and four 3- minute work intervals. Work:rest ratios decreased from 1:2 to 1:1.3. In addition to the interval training sessions, subjects performed one continuous training session of cycling on rollers or windtrainers at an intensity of 20-25 bpm below T_{vent} for 60 minutes once per week during the I phase.

The U phase consisted of four days of performance testing, four required rest days, one session of continuous cycling for 40 minutes at 5 bpm below T_{vent}, and one interval session (six 2- minute exercise intervals).

During the V and I phases only, subjects completed one resistance training session per week in order to maintain initial strength. The strength workouts involved resistive exercise with free weights and plate loaded machines for the following movements: bilateral leg press, unilateral knee extension and flexion, calf raises, bench press, shoulder press, arm curl, and triceps extension. The repetitions and sets ranged from 6 - 12 and 3 - 5, respectively, and intensity varied from 70 - 85% of 1 repetition maximum (1RM). The strength training program was periodized using a computer software program (B.E. Software, Lincoln, NE).

3.2.4 Immune and Hormone Analysis

The immune factors measured were leukocyte counts, lymphocyte subset counts, and serum concentrations of the cytokines IL-1β, IL-6, and TNFα. The hormone factors measured were serum testosterone and urinary cortisol concentrations.

Lymphocyte subset counts were made on fresh EDTA-anticoagulated venous blood. Venous samples were drawn from an antecubital vein at a consistent specified time between 7:00 a.m. and 9:00 a.m. for each subject, after refraining from exercise for 36 to 44 hours. Urinary cortisol concentration was measured from pooled 24 hour urine collections. Urine was collected over a 24 hour period of no exercise which started 24 - 26 hours after the last training session. The collected serum and well mixed, 24 hour urine sample were frozen at -80°C until assays were performed.

The lymphocyte subset counts were measured by dual fluorescence, lyzed whole blood protocol using Simultest reagents (Becton Dickinson, San Jose, CA, USA) containing antibody pairs directly conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE). The Simultest reagents included the following: CD45-FITC/CD14-PE, CD3-FITC/CD4-PE, CD3-FITC/CD8-PE, CD3-FITC/CD19-PE, and CD3-FITC/CD16, 56-PE. Antibody-labeled cells were analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). The monoclonal antibodies were specific for total T cells (CD3+), T helper/inducer cells (CD3+CD4+), T suppressor/cytolytic cells (CD3+CD8+), total B cells (CD19+), and natural killer cells (CD3-CD16+CD56+).

Cytokine concentrations were determined using commercial enzyme-linked immunosorbent sandwich assay (ELISA) kits (Medgenix Diagnostics SA, Fleurus, Belgium). Manufacturer reported intra-assay coefficients of variation for the IL-1β, IL-6, and TNFα kits were 2.2 - 3.4, 4.7 - 5.6%, and 1.4 - 5.2% respectively, and inter-assay coefficients of variation for each of the kits were 4.4 - 4.6%, 2.20 - 7.49%, and 8.0 - 9.9%.

Serum testosterone and 24 hour urinary free cortisol concentrations were determined using ^{125}I radioimmunoassay kits (Clinical Assays™ Gamma Coat™, Incstar Corporation, Stillwater, Minnesota, USA). Manufacturer reported intra-assay coefficients of variation for the testosterone and cortisol kits were 6.2 - 8.6% and 6.6 - 7.7% respectively, and inter-assay coefficients of variation were 6.9 - 13.6% and 8.8 - 9.8% respectively.

3.2.5 Physiological and Performance Tests

$\dot{V}O_{2max}$ and power output at T_{vent} (POT_{vent}), were measured during a stepwise incremental maximal cycle ergometer (Monark 818E) test following a 5 minute warm up at 60 - 90 Watts (W). A pedaling cadence of 80 rpm was maintained throughout the test. Increments of ~20 W were added every three minutes until T_{vent} was visually identified as a distinct increase in the ventilatory equivalent for carbon dioxide ($\dot{V}_E / \dot{V}CO_2$). Please refer to Appendix B for further description on the determination of T_{vent}. Once T_{vent} was clearly established, the power output was increased ~20 W each minute until volitional fatigue. T_{vent} was taken as the power output prior to the exercise load which caused the systematic increase in the $\dot{V}_E / \dot{V}CO_2$ ratio. Two investigators (SML and SP) agreed on final determinations of T_{vent} from the data following the tests. The peak 20s $\dot{V}O_2$ value was recorded as $\dot{V}O_{2max}$, provided that either: there was <0.10 l·min^{-1} increase in $\dot{V}O_2$ when power output was increased; the respiratory exchange ratio (RER) > 1.10; or the subject was too fatigued to continue (56). Expired gases were continuously sampled, averaged, and recorded every 20 seconds using a SensorMedics 2900Z metabolic measurement cart (MMC) (SensorMedics, CA, USA). Heart rates were continuously monitored using an electrocardiograph (ECG) monitor (Hewlet Packard, USA) or a heart rate monitor (Polar Electro, Finland) that was checked for accuracy against an ECG.

After 24 hours of rest, cycling economy was assessed from the measurement of oxygen consumption ($\dot{V}O_2$) and heart rate (HR) during three consecutive 6 minute submaximal loads of approximately 50, 70, and 90 percent of T_{vent} on a Monark 818E cycle ergometer. $\dot{V}O_2$ and HR at a power output of 200 W were determined by regressing the values for $\dot{V}O_2$ and HR against the power outputs for each of the three submaximal loads, obtaining the slope of the line, and solving for $\dot{V}O_2$ and HR at 200 W. This power output was chosen because it fell between the 50 and 90 % of T_{vent} workloads for all subjects throughout all of the testing periods. At baseline, V, I, and U, 200 W was 74, 65, 64, and 63 % of T_{vent}, respectively. $\dot{V}O_2$ and HR were recorded in the manner previously described. Cycling economy was calculated by this method as opposed to being measured at a power output of 200 W because a single oxygen cost for one power output may fail to account for decrements (or improvements) in an athlete's economy over a range of power outputs (57). Economy should be described as the athlete's total response to a series of submaximal workloads (57, 58).

Within 20 to 30 minutes following the cycling economy test, a simulated 20 km time trial was used to measure cycling performance, as previously described by Norris et al. (59). Subjects used their personal bicycles (consistent equipment and tire pressure) on a Kreitler "Killer Headwind" front fork supported roller system (Kansas City, Mi., USA). Fan resistance of the roller system was maintained at a constant setting for each trial throughout the study. An electronic counter was attached to the rear roller and interfaced with a personal computer equipped with a custom designed software program to calculate distance, time, and velocity. Subjects were allowed to watch the computer display of cycling speed and distance traveled, but were not given feedback regarding the elapsed time of testing.

3.2.6 Statistical analysis

One way ANOVA with repeated measures was used to analyze the effect of training on dependent variables. If the observed F-ratio was statistically significant ($p < 0.05$) a Scheffé post hoc test was used to locate the differences. Alpha for the repeated measures ANOVA and Scheffé was set at $p < 0.05$.

3.3 RESULTS

3.3.1 Clinical information.

Of the 9 subjects who complied fully with the study protocol, there were 4 cases of mild colds (e.g., rhinitis, sinusitis): two cases during B, none during V, and one case each during I and U. In addition, one subject had a moderate case of flu lasting one week during V. This subject halted training for one week, but resumed after one week of recovery and finished the study one week after the rest of the group.

3.3.2 Immune Factors.

There were no significant changes in lymphocyte subset counts, IL-1β, IL-6, or TNFα after V1, V2, I1, I2, or U, as compared to baseline (Tables 3 - 2 and 3 - 3). Immune cell counts (47) and cytokine (60) concentrations were within normal ranges.

3.3.3 Hormonal Factors.

There were no significant changes in cortisol or testosterone concentrations after V1, V2, I1, I2, or U, as compared to baseline (Table 3 - 4). Cortisol and testosterone concentrations were within normal ranges (61, 62).

3.3.4 Physiological and Performance Tests.

3.3.4.1 $\dot{V}O_{2max}$

$\dot{V}O_{2max}$ increased significantly by 5.8 % from 4.46 ± 0.39 $l{\cdot}min^{-1}$ at B to 4.72 ± 0.36 $l{\cdot}min^{-1}$ after V. $\dot{V}O_{2max}$ remained significantly higher, compared to B, following I (6.7 %) and at U (9.4 %). There were no significant differences in $\dot{V}O_{2max}$ between V, I, and U (Table 3 - 5).

3.3.4.2 POT_{vent}

POT_{vent} increased significantly by 14.1 % from 269 ± 35.5 W at B to 307 ± 31.6 W following V. POT_{vent} remained significantly higher, compared to B, following I (15.6 %) and at U (19.0 %). There were no significant differences in POT_{vent} between V, I, and U (Table 3 - 6).

3.3.4.3 Cycling Economy

Economy, as measured by $\dot{V}O_2$ and HR at 200 W, improved significantly from 2.95 $l \cdot min^{-1}$ and 152 bpm at B to 2.76 $l \cdot min^{-1}$ (6.9 %) and 136 bpm (11.8 %) after V. Economy, as measured by $\dot{V}O_2$, remained significantly improved, compared to B, following I (3.9 %) and at U (4.6 %). There were no significant differences in economy measured as $\dot{V}O_2$ between V, I, and U. Economy, as measured by HR, remained significantly improved, compared to B, following I (9.4 %) and at U (10.9 %). There were no significant differences in economy measured as $\dot{V}O_2$ between V, I, and U (Table 3 - 7).

3.3.4.4 20 km Performance Time

20 km performance time decreased significantly by 5.9 % from 35:15 ± 2:36 min:s at B to 33:18 ± 2:27 min:s following V, and further decreased significantly by 2.1 % to 32:38 ± 2:26 min:s after U. However, there were no significant differences in 20 km performance time between V and I, or between I and U (Table 3 - 8).

3.4 DISCUSSION

The hypothesis that nine weeks of continuous and interval cycle training would alter immune status, as indicated by reduced lymphocyte subset counts and IL-1β, IL-6, and TNFα concentrations, was not supported by the results of this study. Furthermore, resting urinary cortisol excretion and serum testosterone concentrations (determined 24 - 44 hours after exercise) were not significantly altered. Therefore, the athletes completed nine weeks of strenuous training sufficient to increase their endurance fitness in the absence of any detectable changes in the immune system.

3.4.1 Physiological and Performance Tests

Superior performance in $\dot{V}O_{2max}$, POT_{vent}, cycling economy, and time trial tests are all important for endurance cycling success (63, 64). These performance variables were monitored in order to quantify the fitness improvements elicited by the training program. The improvements observed in the physiological and performance tests were of the magnitude expected considering the training program undertaken. Several recent studies reporting immune responses to endurance training stress have not included performance results (7, 8, 11). This is a weakness of these particular studies since it is difficult to assess the nature of the training program.

3.4.1.1 $\dot{V}O_{2max}$

$\dot{V}O_{2max}$ significantly increased by 5.8 % above baseline values after volume (V) training, and the improvements were maintained through the I (6.7 %) and U (9.4 %) phases. These improvements are consistent with those reported by others (43, 49, 50, 65). After four weeks of a similar training program with similar subjects, Norris (49) reported that $\dot{V}O_{2max}$ had improved by 5 %. No further changes were seen after an additional four weeks of training. In both studies, subjects were competitive cyclists of similar age, with similar $\dot{V}O_{2max}$ values at the onset of training (56.8 ± 6.6 $ml \cdot min^{-1} \cdot kg^{-1}$ (49) compared to 58.7 ± 5.4 $ml \cdot min^{-1} \cdot kg^{-1}$). As well, endurance training intensity was set at and monitored using heart rate at ventilatory threshold. An important difference between the studies was that Norris (49) apparently used a more conservative estimate of T_{vent} which would tend to reduce the training intensity for his subjects. Norris (49) reported that ventilatory threshold (2.61 $l \cdot min^{-1}$) occurred at 65 % of $\dot{V}O_{2max}$, whereas in the present study $\dot{V}O_2$ at T_{vent} (3.56 $l \cdot min^{-1}$) occurred at 79 % of $\dot{V}O_{2max}$. Furthermore, in the present study, the endurance training sessions were longer in duration (50 - 75 vs. 40 - 55 minutes). Finally, it should be noted that high intensity interval training occurred in the present study and not in the study by Norris (49).

Neary (50) reported a 12 % increase in $\dot{V}O_{2max}$ in club level competitive cyclists and triathletes, who performed endurance cycle training for 7 weeks. The first two weeks of the training program entailed 4 $d \cdot wk^{-1}$, 45 $min \cdot session^{-1}$ cycling at an intensity just below T_{vent} (80 % HRmax). The final five weeks consisted of 5 $d \cdot wk^{-1}$, 60 $min \cdot session^{-1}$, intensity at or slightly above T_{vent} (90 % HR_{max}). Initial fitness was lower in the subjects in Neary's study ($\dot{V}O_{2max}$ ± SD = 52.5 ± 1.1 $ml \cdot min^{-1} \cdot kg^{-1}$ (50) vs 58.7 ± 5.4 $ml \cdot min^{-1} \cdot kg^{-1}$), which may help explain the greater improvement. It has been suggested that improvements in $\dot{V}O_{2max}$ are more difficult to obtain with increased fitness (66). Training intensities were similar, as the subjects in Neary (50) trained for 5 weeks at heart rates of 90% of HR_{max}. In the present study, endurance cycle session heart rates averaged between 85 - 93% of HR_{max}. Lopez Calbet et al. (43) reported no significant improvements in $\dot{V}O_{2max}$ in 7 highly trained cyclists ($\dot{V}O_{2max}$ = 75.5 ± 4.8 $ml \cdot min^{-1} \cdot kg^{-1}$) after six months of competitive cycling. Barbeau et al. (67) also reported no improvement in $\dot{V}O_{2max}$ in elite male cyclists ($\dot{V}O_{2max}$ = 69.0 ± 4.9 $ml \cdot min^{-1} \cdot kg^{-1}$) following 5 months of cycle training and elite competition.

In summary, the increased $\dot{V}O_{2max}$ was consistent with expectations relative to initial fitness and the training program imposed. Subjects who had lower fitness experienced greater improvements in $\dot{V}O_{2max}$ with endurance cycle training (50, 65). Subjects who were similar in fitness, but underwent a slightly easier training program experienced slightly less, but significantly improved, increases in $\dot{V}O_{2max}$ (49). Subjects with greater fitness, who underwent harder and longer endurance cycling programs did not experience significant improvements in $\dot{V}O_{2max}$ even when endurance training stress was greatly increased (43, 67).

$\dot{V}O_{2max}$ has been considered one of the best indicators of endurance potential (68). However, it has been demonstrated that $\dot{V}O_{2max}$ can have a limited predictive value for endurance performance in homogeneous groups of high-performance athletes (49, 64). For this reason, additional physiological tests, such as anaerobic threshold, economy, and performance trials should also be used to evaluate fitness and predict competitive success (63, 64).

3.4.1.2 Power Output at Ventilatory Threshold (POT_{vent})

POT_{vent} increased by 14.1% in 6 weeks (at V), and increased to 15.6 % and 19.0 % above baseline values at I and U, respectively. These results are consistent with other reports (43, 49, 50). Lopez Calbet et al (43) reported smaller increases (11.4 % in 6 months (43) vs. 19.0 % in 10.5 weeks) in POT_{vent} in elite cyclists. Neary (50) reported a similar increase in POT_{vent} (16.3 % in 7 weeks). Norris (49) reported a 16.5 % increase in POT_{vent} (W) after four weeks of training, and a 27 % increase above baseline values by eight weeks of training. Norris reported greater improvements despite a less demanding program. The determination of T_{vent} by Norris was more conservative than that of Lopez Calbet et al. (43) and of the present study. The use of different thresholds makes the comparison of the POT_{vent} values difficult because the physiological processes underlying the thresholds are different, although the processes are proposed to be related (49, 69). Interestingly, Lopez Calbet et al. (43) showed that both threshold points improved by similar amounts following training. In this investigation, power output at ventilatory threshold #1 (increase in $\dot{V}_E / \dot{V}O_2$ without an increase in $\dot{V}_E / \dot{V}CO_2$) and ventilatory threshold #2 (non-linear increase in $\dot{V}_E / \dot{V}CO_2$) were measured. Following the six month training period, POT_{vent}#1 increased by 11.1% and POT_{vent}#2 increased by 11.4%.

3.4.1.3 Cycling Economy.

Barbeau et al. (67) suggested that heart rate and oxygen consumption at a given submaximal load could be a better indicator of training status of elite cyclists than $\dot{V}O_{2max}$. These authors postulated that in experienced cyclists, peripheral adaptations in the working muscles may be far more important for enhanced submaximal working capacity than central adaptations (67). The subjects in the present study were not, as a group, elite, however the assessment of economy is still an important training status. As well, it has been suggested that decreased economy reflects accumulated training stress (70, 73). This parameter was therefore of significant interest in the present investigation.

Improvements in cycling economy in the present study were supported by those reported by other authors. Cycling economy, as measured by $\dot{V}O_2$ and HR at 200 W improved by 6.9 % and 11.8 % respectively at V as compared to B. Lopez Calbet et al.

(43) reported that $\dot{V}O_2$ at five sub-threshold power outputs (200, 220, 240, 260, 280 W) significantly decreased at all power outputs, except at 280 W, following 6 months of competition. At 240 W, the reported decrease in $\dot{V}O_2$ was 7%, from 3.59 $l \cdot min^{-1}$ to 3.344 $l \cdot min^{-1}$. A similar finding was reported by Barbeau et al. (67). $\dot{V}O_2$ significantly decreased in elite competitive cyclists at 200 W after volume training, and at 250 W when comparing post-volume training to mid-competitive season measurements. HR at 200 and 250 W significantly decreased when pre- to post- high volume training measurements were compared.

3.4.1.4 Simulated Time Trial Performance.

Performance time for simulated 20 km races significantly decreased by 5.9 % at V compared to B. At I and U, 20 km performance time had remained significantly decreased by 6.7 % and 8.1% respectively compared to B. Additionally, 20 km time at U had significantly improved (2.1 %) as compared to V. Neary (50) reported a 15 % decrease in 40 km time trial time after 7 weeks of training. Norris (49) reported a 7.4 % decrease in 40 km time trial after 4 weeks, and a total 9.1 % decrease after the full 8 week period. Neary (50) and Norris (49) conducted time trial tests on an indoor roller system, similar to the system used in the present study. The improvements in time trial performance reported by Neary (50) were greater than those reported by the present study. The subjects in Neary (50) were less fit at the onset of the study, and therefore had greater potential for improvement. Although, improvements in a 40 km time trial may not necessarily be directly compared to those reported for a 20 km time trial as they are different distances and thus are different races.

3.4.2. Training Stress

While it is difficult to quantify training stress, the training regimen in this study can be considered as difficult. Although subjects during their competitive season may train and compete at a greater duration, frequency, and intensity than what was performed in this study, this study was performed in the preparatory season period, commencing following transition season, where little training was performed by the majority of the subjects. Training was strictly controlled to ensure adherence, therefore subjects could not ride their bicycles outdoors. Subjects rode bicycles indoors on rollers, often in a fairly warm room, sometimes with only the company of the supervising researcher. Such training is often repetitive and boring, which could increase the level of stress perceived by the subjects.

Training intensity was monitored using heart rate monitors. Heart rates would likely have risen due to cardiovascular drift during training sessions (71). Maintenance of a target heart rate could have the effect of reducing absolute work intensity towards the end of a training session. Continuous cycle training session intensity was measured and monitored using heart rates, as opposed to power outputs, because subjects rode their personal bicycles on rollers during training sessions. Determining power outputs for the bicycles and rollers was not possible as neither were originally constructed to be calibrated. Training could have been completed on Monark cycle ergometers, which would have allowed the training intensity prescriptions to be given as power outputs, but it is questionable whether the subjects would have been agreeable to performing endurance cycle rides almost daily on cycle ergometers for a 9 week period. However, although it was not possible to assess absolute intensity, use of heart rate to monitor exercise intensity allowed relative intensity to remain constant as fitness increased. It can be assumed that absolute intensity increased as fitness increased. Additionally, with training, submaximal heart rates decrease, and if training prescription heart rates are not re-established for the new fitness level, subjects will train at higher relative intensities (67). This was the case for several of the subjects. Within two weeks after the start of training, most of the subjects found it harder to maintain their prescribed training heart rates, and had to cycle at harder intensities to maintain their training at the prescribed level.

The subjects did perform strength training on one day per week throughout the study. It has been reported that there may be an interference effect between the two types of training. Usually, when both resistance training and endurance training are performed concurrently for extended periods, strength development is inhibited but aerobic fitness is not (72). The fact that no significant changes in any of the immune or hormonal indicators were seen suggests that the strength training was not a factor in causing alterations in the independent variables. The strength training stimulus could possibly have had a masking effect, though, on the immune and hormone variable. The strength training sessions were selectively added to the training regimen because subjects did not want to lose any of their initial muscular strength. It was desirable to control the strength training rather than allow subjects to train independently on their own. Therefore, a consistent strength training session was implemented for all subjects one day per week during V and I.

The training program was a regimented physical stress that none of the subjects were accustomed to in the weeks prior to the onset of the study. The training program was about as hard as the subjects were willing to participate in. It was designed to be a significant new stress imposed on the subjects. However, it was not a great enough stress to cause overreaching, as defined by a fall in performance ability that did not return to baseline values following a reasonable regeneration period (17, 73). The purpose of the training program was not to cause overreaching but to impose a heavy endurance training stress.

3.4.3 Leukocyte and Lymphocyte Subset Counts

The lack of significant alterations in leukocyte or lymphocyte subset counts in longitudinal training studies is becoming a common result (6, 8, 9, 11, 13, 74, 75). This study found no significant alterations in any leukocyte or lymphocyte subset count. Wolfarth et al. (13) reported that there were no alterations in CD3, CD4, CD8, or CD16 counts were found following an 8 week intense training phase in 16 top class endurance cross-country skiers and cyclists. Lötzerich (74) reported that throughout a complete track and field season, 11 female short distance runners showed no significant changes in any leukocyte or lymphocyte count and concluded that the immune system stable throughout the season. Kajiura et al. (8) found no changes in resting lymphocyte subset percentages over forty days of alternating 10 day periods of heavy and easier endurance training in young male recreational and competitive athletes. Generally, the emerging literature is supportive of our finding of no significant alterations in leukocyte or lymphocyte subset counts. However, several studies have shown significant alterations in immune cell counts. Several studies have reported alterations in only one cell type out of a panel of immune cell counts. Gabriel et al. (75) reported a significant decrease in eosinophils only during training or overtraining conditions in elite endurance athletes. Baum et al. (11) reported stability in leukocyte and lymphocyte counts, except for a significant increase in monocytes during a season of training and competition in 20 competitive middle distance runners. Verde et al. (9) studied distance runners and reported a significant reduction in resting CD4:CD8 ratio after three weeks of training at 125 % of baseline distance. After a further three weeks of training at the original baseline level, the CD4:CD8 ratio had returned to normal. Fry et al. (6) reported significantly lower natural killer (CD56) lymphocyte levels during, at the end of, and five days after 10 days of overload training consisting of twice daily interval training. Several studies have reported that counts of more than one lymphocyte cell type have been altered after endurance training and competition. Pizza et al. (7) found that lymphocyte subset counts were measured over two 10 day overloading phases, which consisted of 200% of normal training volume, interspersed with reduced training (80% of normal training) in moderately trained male runners. Baj et al. (10) reported that absolute lymphocyte, CD3, CD4, and CD8 counts were significantly depressed after 10 days of the overload training when compared to pre-training values while CD16 counts were unchanged. Significantly decreased absolute numbers of CD3 and CD4 lymphocytes and a decreased CD4:CD8 ratio were reported in elite competitive cyclists when comparing before and after 6 months of intensive training and a racing season, during which subjects cycled approximately 500 km a week. These examples show the inconsistent effects of endurance training stress on immune cell counts in athletes. More research is required to determine how an endurance training stress affects leukocyte and lymphocyte subset counts.

Similar to the present study, the studies just cited utilized flow cytometry to measure immune cell counts, used competitive athletes as subjects, and monitored subjects over stressful training and/or competition phases that imposed an increased exercise stress on the subjects. It is difficult to determine why the few longitudinal studies which have measured leukocyte and lymphocyte subset counts have found such variable results. Studies which have measured immune cell counts in previous untrained subjects undertaking moderate training have also had varying results. In one such study, no significant alterations in lymphocyte subset counts were reported when 9 subjects completed a moderate intensity six week, every second day, 45 minute per session training program (76). Another study reported that the lymphocyte subset count populations of CD2, CD4, CD8, and CD20 increased significantly following a 10 week program consisting of three 45 minutes session of cycle ergometry per week at 70 - 80% of age-predicted heart rate maximum in untrained males (77).

The inconsistency of these results may be due to differences in sampling time and/or training regimen. Our study, while looking particularly for chronic effects, attempted to rule out the effects of acute exercise by ensuring 36 - 44 hours of no exercise before drawing the samples. Reported training regimens have been highly variable. We monitored immune parameters over 9 weeks of progressive hard training, while others (6, 7, 9) involved only 10 to 21 day bouts of very intensive work outs, or followed athletes over a full competitive season (10, 11).

An additional possibility to explain the inconsistent results is that lymphocyte subset counts may not be a sensitive marker of immune status. Verde et al. (9) proposed that simple blood counts (lymphocyte subsets) can give misleading information about immune function. Their results showed a decrease in T cell proliferation as assessed by phytohemagglutinin and concanavalin stimulation, while their lymphocyte subset counts showed no change. Alternatively, Baj et al. (10) found significant decreases in absolute counts of CD3, CD4, and in the CD4:CD8 ratio, but not in CD8 and CD16 cells after 6 months of intensive cycle training and racing in young cyclists, whereas lymphocyte proliferation was found to be elevated when induced by phytohemagglutin (PHA) and anti-CD3 monoclonal antibodies. These studies (9, 10) demonstrate inconsistent findings between lymphocyte subset counts and lymphocyte proliferation rates.

It is possible that although counts of specific immune cells may, or may not, be altered by endurance training, there may be a change in the number or percentage of immune cells which express surface molecules indicating recent cell. Research has shown that while leukocyte and lymphocyte subset counts were generally not altered, indicators that these cells had experienced higher levels of activation were apparent (6, 10, 11). For example, Fry et al. (6) reported that although T_{helper} (CD4) cell counts were not altered during, at the end of, and five days after 10 days of overload training consisting of twice daily interval training, but the number of activated T_{helper} (CD25) cell were significantly increased. Also, T cell activation levels measured by another method, represented by HLA-DR+ counts, were also elevated at the end of, and five days after 10 days of overload training. The present study did not measure any indicators of immune cell activity, therefore it is not known if the immune cells of the subjects had been more active but had not changed in number.

3.4.4 Cytokines

Our results showed no significant changes in serum IL-1β, IL-6, or TNFα cytokine concentrations. This finding is supported by Kayashima et al. (12) who reported no significant changes in serum TNFα and IL-1β concentrations following a 4 week period of severe endurance training stress. However, Horne et al. (25) did report that plasma TNFα rose during a combined endurance and strength training program in female but not in male subjects. These are the very few longitudinal studies which have monitored cytokines during periods of training. Cytokines are protein molecules that mediate the effector phases of both natural and specific immunity. The principal function of IL-1 and of TNFα is to mediate the host inflammatory response in natural immunity. IL-1, TNFα, and IL-6 play important roles in the acute phase response; they act on hepatocytes to secrete serum proteins important in the acute phase response (26). Significant increases in IL-1, TNFα, and IL-6 have been reported following strenuous exercise (78, 79). Muscle and liver damage induced by chronic strenuous physical exercise (12) may play a role in eliciting the acute phase response. Zhou et al. (24) suggested that IL-6 may be released into plasma under the regulation of neural and endocrine responses to stress and that IL-6 may induce acute-phase proteins and glucocorticoid production, thus contributing to the hormonal response to exercise. The result of no significant changes in resting concentrations of cytokines in the present study suggests that, at rest, the subjects had not experienced a level of stress which would cause cytokine concentrations to be elevated. Cytokine concentrations could have returned to normal following individual acute training sessions. Subjects could have become accustomed to the training load, and were not experiencing ongoing muscle damage which could cause an acute phase response.

The cytokine concentrations were highly variable. For IL-6, the standard deviation values were larger than the mean concentrations. Serum IL-6 values seemed, anecdotally, to be slightly elevated in subjects who were experiencing colds. Figure 3 - 2 displays individual IL-6 results. The 400 - 500% increase in specific individual results account for the high standard deviations. With the low subject number, the large difference in IL-6 values between subjects resulted in high standard deviations values. Two of these three dramatic increases in IL-6 concentration occurred concomitantly with infectious episodes. All of the serum cytokine concentrations were within ranges reported in healthy subjects by the manufacturers of the biochemical assay kits utilized to assess cytokine concentrations (60). Examples from other clinical studies indicate that the IL-6 concentrations (ranged from 0.8 - 1.9 $pg \cdot ml^{-1}$) were well within normal control values. Southern et al. (80) reported that clinically symptom-free men, median age 50 (range 46 - 72), had average serum IL-6 values of 1.66 to 5.38 $pg \cdot ml^{-1}$. Spadaro et al. (81) reported that healthy control values for serum IL-6 were 2.8 ± 0.7 $pg \cdot ml^{-1}$, patients with psoriatic arthritis had values of 52.4 ± 12.2 $pg \cdot ml^{-1}$, patients with rheumatoid arthritis had values of 150.4 ± 39.8 $pg \cdot ml^{-1}$, and those with psoriasis had values of 12.6 ± 4.9 $pg \cdot ml^{-1}$. Krafte-Jacobs and Bock (82) reported that children critically ill with sepsis had plasma IL-6 concentrations of 688 ± 258 $pg \cdot ml^{-1}$, and children with septic shock had values of 27,469 ± 13,647 $pg \cdot ml^{-1}$. Control children had plasma IL-6 values of 7 ± 1 $pg \cdot ml^{-1}$. Cohick et al. (83) reported that mean normal serum IL-6 concentrations were 3 $pg \cdot ml^{-1}$ and that rheumatoid arthritis patients had a mean serum IL-6 concentration of 36 $pg \cdot ml^{-1}$ (range 12 - 403). The type of infection plays a role in the IL-6 response to infection, which is to be expected. Smith et al. (84) reported that only 24% of patients with community acquired pneumonia had detectable plasma IL-6 levels during their hospital stay. Thea et al. (85) reported that plasma levels of IL-6 were undetectable in all but 3 of 48 patients with advanced AIDS, in 2 of 51 patients who were asymptomatic HIV+, and in 4 of 11 HIV- controls. The results of these studies show that the IL-6 values of the present study were within normal values and that the large variability in IL-6 values was clinically insignificant.

The dependence on the type of infection, and thus the type of immune response illustrates the need to precisely choose the immune markers which would be relevant to the immune response elicited during the stress of endurance training. IL-6 may be a good choice of cytokine to measure as it is active in the acute phase response activated in response to muscle damage. The TNFα (8.4 - 11.2 $pg \cdot ml^{-1}$) and IL-1β (4.9 - 6.0 $pg \cdot ml^{-1}$) values were all within normal ranges for healthy controls (60). Thea et al. (85) reported that HIV- healthy controls had below detection limit plasma TNFα and Il-1β values. Only 4 out of 48 patients with advanced AIDS has detectable TNFα and 2 out of 48 had detectable IL-1β values. HIV+ asymptomatic patients had a plasma TNFα median value of 210 pg·ml^{-1} (range 78 - 370 pg·ml^{-1}) and an IL-1β median value of 320 pg·ml^{-1} (range 156 - 800 pg·ml^{-1}). Adami et al. (86) reported that normal control values of serum TNFα was 0.11 ± 0.08 pg·ml^{-1}. Patients with B-cell chronic lymphocytic leukaemia had serum TNFα values of 36 ± 5 pg·ml^{-1}. Patients with typhoid fever had whole blood IL-1β values less than 140 pg·ml^{-1}, IL-6 values of 96 ± 131 pg·ml^{-1}, and TNFα values of 130 ±50 pg·ml^{-1} (87). These examples give an idea of the circulating concentrations of these cytokines in clinical conditions. The concentrations of IL-1β, IL-6, and TNFα in this study were not in the range of clinically significant values seen in the type of infection noted here.

3.4.5 Urinary Free Cortisol

The lack of significant change in resting 24 hour urinary free cortisol concentration suggests that the endurance training did not induce a chronic stress response in the cyclists. Elevated resting serum cortisol levels were found in swimmers who doubled their training distance while maintaining intensity (at ~95% VO_{2max}) for 10 days (31). Neary et al. (88) found that resting 24 hour urinary cortisol rose after the third week of cycle endurance training and remained elevated until after training, during which VO_{2max} significantly increased after 7 weeks of training. In this study, 24 hour urine collection began in the morning after an evening training session (personal communication), whereas in the present study a full 24 hours elapsed before commencing the 24 hour urine collection. On the other hand, Lehmann et al. (45) observed that 24 hour urinary cortisol excretion fell to 70% of its baseline value during their study designed to elicit an overtraining syndrome in eight experienced middle- and long-distance runners. Six subjects experienced a decrease and two showed no change in their maximum work capacity, indicated by a decrease in total running distance during incremental treadmill ergometry. Thus, urinary cortisol excretion may rise even when performance has not suffered (88) or fall even when subjects are experiencing excessive training fatigue (45). Our result of no significant change in 24 hour urinary free cortisol is supported by some studies (37, 40, 41, 42, 43) but not by others (30, 31, 32, 33, 34, 35, 36). It may be that resting cortisol values are not good indicators of training stress, as suggested by Hooper and MacKinnon (29), or that training stress was insufficient to elicit a stress response which would cause elevations in resting cortisol concentrations.

3.4.6 Serum Testosterone

Stress, physical or emotional, has long been recognized as a profound disruptive factor in reproductive function (28, 89). Endurance training has been reported to have negative effects on testosterone production. The results of this study indicated that no alterations in testosterone concentration occurred in conjunction with increased training stress. Decreases in testosterone concentrations were seen in endurance athletes undergoing rigorous training in longitudinal studies (37, 38, 39, 90). Alternatively, several studies have shown no changes in testosterone concentrations over longitudinal periods of intense training (6, 35, 43, 44). A cross-sectional study showed endurance trained athletes to have lower circulating testosterone levels than untrained subjects (91). As with measuring cortisol as an indicator of training stress, either the measurement of testosterone may not be a sensitive indicator of training stress, or our subjects may not have trained hard enough to see depressions in circulating testosterone levels.

3.4.7 Proposed Mechanisms Underlying the Training-Immune Link

A short discussion follows on the feasibility of the mechanisms felt to potentially be involved in the hypothesized link between endurance training and immune function.

3.4.7.1 Stress Response

One proposed mechanism for suppression of lymphocyte subset counts during endurance training was that of a chronic stress response (92). The suggestion was that glucocorticoids, secreted in response to the heavy endurance training stress, would have a suppressive effect on the immune system. It is well known that cortisol has both immunosuppressive and anti-inflammatory actions (93, 94, 95). In the present study, urinary free cortisol concentrations were not elevated and no immune marker was significantly altered by the endurance training regimen imposed. It would appear that one of the potential reasons why immune cell counts or that cytokine concentrations were not altered was that the endurance training was not stressful enough to elicit an elevation in cortisol secretion from the adrenal glands above average levels.

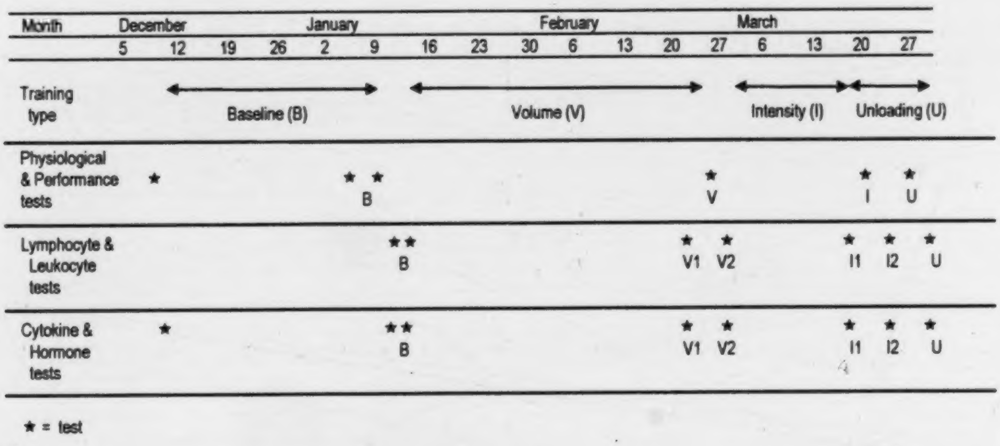
3.4.7.2 Muscle Damage Causes an Acute Phase Response.

A second proposed mechanism explaining exercise-altered immune function is that exercise induced muscle damage elicits an acute phase response characterized by elevated circulating cytokine concentrations such as IL-6, TNFα, and IL-1β. As well, leukocyte and lymphocyte subset counts could be altered as a result of being recruited into muscle tissue which has been damaged as a result of strenuous exercise. No significant changes in any of these variables occurred, and all values were within clinically normal values, therefore it would appear that at rest the immune system was not altered from normal values by training induced muscle damage.

3.4.8 Conclusion

It is concluded that the type of training performed by our subjects did not induce overtraining or immunosuppression. Present results suggest that the immune status as measured by whole blood lymphocyte subsets and serum cytokine concentrations of healthy cyclists, is robust and able to tolerate endurance training with no apparent adverse effects while performance is increased. A possible explanation why no significant changes were seen in the immunological or hormonal indices could be that the subjects did not train hard enough for changes to be seen. Another potential explanation may be that resting lymphocyte subset counts and concentrations of cytokines are not the most sensitive indicators of the effects of training on immune status. Activation markers on certain lymphocytes, or proliferation rates of cells could be measured. Furthermore, measurements of immunological markers at rest, without the presence of an immunological challenge, may not reflect the functional state of the immune system. Glucocorticoids suppress the translation of transcription factors for molecules relevant in launching and maintaining an immune response (96, 97). An immune system faced with an immunological challenge may be limited in its ability to mount an appropriate response due to exercise-induced elevated systemic glucocorticoid production. Thus, athletes may succumb to viral and bacterial infections during very stressful glucocorticoid-producing exercise challenges.

In summary, resting lymphocyte subset counts, IL-1β, TNFα, IL-6, 24 hour urinary cortisol excretion, and serum testosterone concentration were monitored in nine male cyclists over a nine week period of endurance training and 10 days of unloading. The data indicate that training improved endurance fitness and cycling performance without significantly altering any of the selected immune and hormonal factors.


Month
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Training type
Baseline (B)
Volume (V)
Intensity (I)
Unloading (U)
Physiological & Performance tests
B
V
I
U
Lymphocyte & Leukocyte tests
B
V1
V2
I1
I2
U
Cytokine & Hormone tests
B
V1
V2
I1
I2
U
★ = test

Figure 3 - 1. Temporal organization of the study.

Table 3 - 1. Training heart rates during the volume training phase. The respective number of training sessions performed per week and the duration of training session at the described heart rates are indicated at the bottom of the table.

Subject #	Heart Rate (bpm)				
	T_{vent}	T_{vent} - 5 bpm	T_{vent} + 5 bpm to - 10 bpm	T_{vent} - 15 bpm	T_{vent} - 25 to 20 bpm
1	172	167	177 - 162	157	147 - 152
2	175	170	180 - 165	160	150 - 155
3	176	171	181 - 166	161	151 - 155
4	170	165	175 - 160	155	145 - 150
5	162	157	167 - 152	147	137 - 142
6	175	170	180 - 165	160	150 - 155
7	181	176	186 - 171	166	156 - 161
8	180	175	185 - 170	165	155 - 160
9	178	173	183 - 168	163	153 - 158
mean	175	170	180 - 165	160	150 - 155

Number of training sessions per week at the heart rate described.		2 / week	1 / week	1 / week	1 / week
Duration of training sessions		50 - 75 min	50 - 75 min	70 - 95 min	90 - 180 min

TABLE 3 - 2. Resting immunological data at baseline, post volume training (V1 and V2), post high intensity training (I1 and I2), and post unloading (U).

		Baseline	V1	V2	I1	I2	U
White Blood Cells (10^9 cells $\cdot$ l^{-1})	mean	5.6	5.9	5.9	5.8	5.6	5.8
	SD	± 1.36	± 0.90	± 1.87	± 1.06	± 0.99	± 1.06
Granulocytes (10^9 cells $\cdot$ l^{-1})$^{\delta}$	mean	2.9	3.0	3.2	3.1	3.1	3.0
	SD	± 0.90	± 0.71	± 1.34	± 0.83	± 0.59	± 0.73
Lymphocytes (10^9 cells $\cdot$ l^{-1})	mean	2.18	2.19	2.08	2.21	2.06	2.31
	SD	± 0.63	± 0.70	± 0.70	± 0.65	± 0.60	± 0.52
Monocytes (10^9 cells $\cdot$ l^{-1})	mean	0.54	0.66	0.66	0.57	0.57	0.54
	SD	± 0.15	± 0.34	± 0.28	± 0.13	± 0.15	± 0.10
$CD3^+$ (10^9 cells $\cdot$ l^{-1})	mean	1.57	1.53	1.50	1.59	1.49	1.68
	SD	± 0.45	± 0.48	± 0.57	± 0.52	±0.44	± 0.41
$CD3^+$ $CD4^+$ (10^9 cells $\cdot$ l^{-1})	mean	0.89	0.88	0.82	0.87	0.85	0.90
	SD	± 0.17	± 0.27	± 0.17	±0.19	± 0.17	± 0.15
$CD3^+$ $CD8^+$ (10^9 cells $\cdot$ l^{-1})	mean	0.66	0.59	0.60	0.64	0.59	0.67
	SD	±0.38	±0.29	± 0.43	± 0.43	± 0.33	± 0.39
$CD4^+$:$CD8^+$ ratio	mean	1.68	1.66	1.68	1.60	1.70	1.63
	SD	± 0.55	± 0.59	±0.68	± 0.49	± 0.66	±0.63
$CD19^+$ (10^9 cells $\cdot$ l^{-1})	mean	0.28	0.28	0.24	0.27	0.27	0.27
	SD	± 0.20	± 0.20	± 0.19	± 0.24	± 0.21	± 0.17
$CD3^-$ $CD16^+$ $CD56^+$ (10^9 cells $\cdot$ l^{-1})	mean	0.31	0.35	0.34	0.35	0.30	0.34
	SD	± 0.13	± 0.16	±0.18	± 0.12	± 0.09	± 0.13

[δ] The granulocyte cell population was made up of neutrophils, eosinophils, and basophils.

TABLE 3 - 3. Resting cytokine data at baseline, post volume training (V1 and V2), post intensity training (I1 and I2), and post unloading (U).

		Baseline	V1	V2	I1	I2	U
IL-1β (pg · ml^{-1})	mean	6.0	5.3	5.4	5.2	5.4	4.9
	SD	± 4.39	± 3.53	± 2.59	± 3.36	± 2.96	± 2.70
IL-6 (pg · ml^{-1})	mean	0.8	1.1	0.8	1.9	1.4	1.7
	SD	± 1.16	± 2.71	± 0.89	± 2.60	± 2.96	± 2.9
TNFα (pg · ml^{-1})	mean	11.0	10.8	9.6	8.4	9.2	11.2
	SD	± 6.31	± 4.23	± 4.61	± 4.44	± 3.7	± 5.14

TABLE 3 - 4. Resting 24 hour urinary free cortisol and serum testosterone data at baseline, post volume training (V1 and V2), post high intensity training (I1 and I2), and post unloading (U).

		Baseline	V1	V2	I1	I2	U
Cortisol (nmol · $24h^{-1}$)	mean	376	347	314	334	325	270
	SD	± 150	± 168	± 193	± 197	± 142	± 159
Testosterone (nmol · ml^{-1})	mean	14.7	14.1	14.2	14.0	13.3	14.0
	SD	± 4.28	± 3.54	± 3.76	± 3.60	± 4.01	± 4.23

Table 3 - 5. Peak physiological and performance data at baseline, post volume training (V), post high intensity training (I), and post unloading (U).

	Baseline	V	I	U
$\dot{V}O_{2max}$ (l·min^{-1})	4.46 ± 0.41	4.72 * ± 0.36	4.76 * ± 0.30	4.88 * ± 0.31
PO_{max} (W)	375 ± 48.8	398 ± 33.7	400 ± 44.3	410 ± 30.0
HR_{max} (bpm)	191 ± 5.5	189 ± 5.9	189 ± 7.4	189 ± 6.2
RER_{max}	1.19 ± 0.049	1.20 ± 0.040	1.16 ± 0.048	1.16 ± 0.052
$\dot{V}_E$ (l·min^{-1})	188 ± 24.5	189 ± 16.6	194 ± 17.4	191 ± 21.1

* $p<0.05$ significance when compared to baseline.

Table 3 - 6. Ventilatory threshold (T_{vent}) data at baseline, post volume training (V), post high intensity training (I), and post unloading (U).

	Baseline	V	I	U
POT_{vent} (W)	269	307 *	311 *	320 *
	± 35.4	± 31.6	± 25.2	± 28.3
% of PO_{max} (%)	72	77	78	78
	± 8.5	± 4.4	± 7.1	± 8.5
$\dot{V}O_2$ at T_{vent} ($l \cdot min^{-1}$)	3.56	3.91 *	4.03 *	4.06 *
	± 0.48	± 0.32	± 0.17	± 0.24
% of $\dot{V}O_{2max}$ (%)	79	84	85	84
	± 6.6	± 6.5	± 4.0	± 4.8
HR at T_{vent} (bpm)	175	174	176	175
	± 6.0	± 6.9	± 7.8	± 6.1
% of HR_{max} (%)	91	92	93	92
	± 1.7	± 2.9	± 1.9	± 2.1

* $p<0.05$ significance when compared to baseline.

Table 3 - 7. Economy data at baseline, post volume training (V), post high intensity training (I), and post unloading (U).

	Baseline	V	I	U
$\dot{V}O_2$ at 200 W ($l \cdot min^{-1}$)	2.95 ± 0.124	2.76 * ± 0.094	2.84 * ± 0.066	2.82 * ± 0.083
$\dot{V}O_2$ at 200 W, % $\dot{V}O_{2max}$	67 ± 8.6	59 * ± 4.5	60 * ± 4.0	58 * ± 4.3
HR at 200 W (bpm)	152 ± 13.8	136 * ±9.4	139 * ± 10.5	137 * ± 12.0
HR at 200 W, % HR_{max}	79 ± 6.6	73 * ± 5.8	74 * ± 5.0	71 * ± 5.0

* $p<0.05$ significance when compared to baseline.

Table 3 - 8. Simulated 20 km performance time trial data at baseline, post volume training (V), post high intensity training (I), and post unloading (U).

	Baseline	V	I	U
20 km time (min:s)	35:15 ± 2:36	33:18 * ± 2:27	33:02 * ± 2:02	32:36 * $^{\varphi}$ ± 2:26

* $p<0.05$ significance when compared to baseline. $^{\varphi}$ $p<0.05$ significance when compared to V.

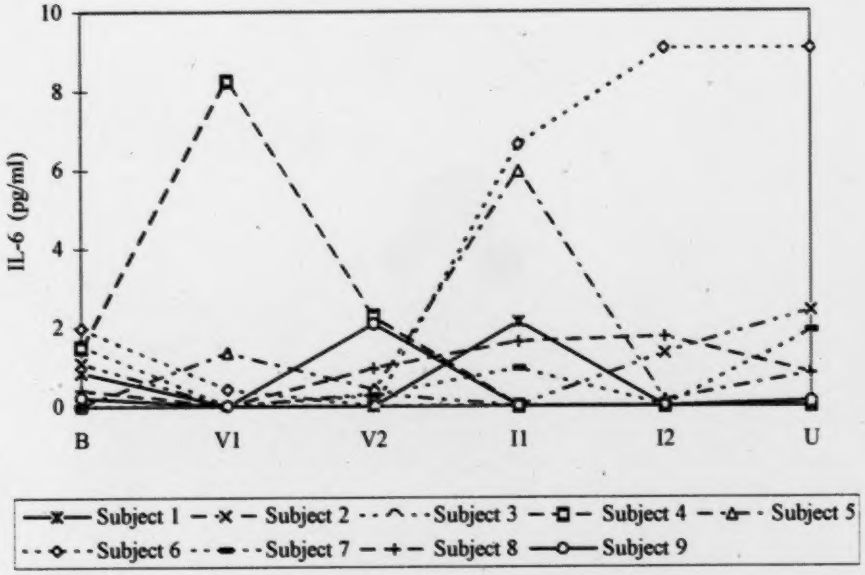

Figure 3 - 2. Individual serum IL-6 concentrations at baseline (B), post volume training (V1 and V2), post high intensity training (I1 and I2), and unloading (U).

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Chapter 4

General Discussion and Conclusions

The purpose of this study was to examine the effects of endurance training with progressive increments in volume and intensity, followed by a short period of reduced volume (unloading), on selected immunological, endocrinological, and performance variables. The hypothesis was that continuous and interval endurance training would be characterized by elevated adrenal cortisol secretion, as shown by 24 hour urinary free cortisol; suppressed reproductive function, characterized by decreased serum testosterone concentrations; and, suppressed immune function, characterized by decreased circulating lymphocyte subset counts and cytokine concentrations. The results of this study did not support this hypothesis as no significant alterations in lymphocyte subset counts or in cytokine concentrations accompanied increases and/or changes in training stress magnitude or type, as imposed by the heavy volume and interval training phases, or by the decreased volume (unloading) training phase. Furthermore, no significant changes were seen in either cortisol or testosterone.

This research has contributed to the field of exercise physiology as it provides evidence that athletes can undertake heavy endurance training without experiencing altered immune cell counts, and altered serum cytokine, urinary cortisol, and serum testosterone concentrations. If cell counts are accurate indicators of immune competence, then the present results support the suggestion that subjects can train hard without compromising immune function.

Further discussion on the magnitude of training stress, on the presence of infectious episodes in the subject population, and on directions for future research will complete the rest of the discussion.

4.1 Magnitude of Training Stress

The prescribed training stress was heavy, although it was not heavy enough to cause an overreaching response, as defined by a decrease in performance values during training which did not return to baseline levels following a reasonable regeneration period (1, 2). In the study, performance measures improved as a result of the training regimen. The training stress did not cause an elevated cortisol excretion, as measured by 24 hour urinary free cortisol. Elevated adrenal cortisol production has been shown to occur as part of a typical hormonal response to stress (3). Thus, it could be concluded that the imposed training stress was not great enough to cause the decreased lymphocyte subset, cytokine, and testosterone measures hypothesized to be due to stress-induced elevated adrenal cortisol production.

It has been shown that during and after acute strenuous exercise, concentrations of circulating lymphocyte subsets (4, 5, 6, 7), cortisol (8), and testosterone (9, 10) are altered from resting values. It would appear that, in this study, the biological measures had returned to resting levels following the specified periods of rest taken before biological samples were obtained. If training stress had been greater, perhaps longer periods of time would have been required for the biological measures to return to resting levels, or new altered resting levels would have developed. In an attempt to rule out the measurement of residual acute exercise-induced alterations in immune cell counts, blood samples were obtained 36 - 44 hours following the last exercise session. The training prescription used in this study was an independent variable, and was not intended to induce an overtrained state. It is most likely that the training intensity or volume of each phase was not great enough to elicit significant changes in the resting immune, cortisol, and testosterone values.

It is interesting to note the perception of the training program by the subjects. The perception of training stress is important in order for the physiological manifestations of stress to be experienced. A stressor can be defined as "any external or internal challenge that disrupts the internal environment" (11). There is a difference between eustress (good) and distress (bad), in that the former involves a positive experience while the latter involves a negative experience (3). For the subjects in this study, the endurance training phases were either an eustress, a distress, or a combination of them both. Exercise is a difficult stressor to quantify, for it is not a homogeneous stressor, but a combination of many physiological and psychological stressors (12). The psychological and physiological aspects must both be considered. It is impossible to know what the effect of the physical stress of exercise alone was on the subjects. No psychological stress profiles or tests were performed on the subjects. The only potential measure of stress level were subject attrition rates and subject compliance to perform the prescribed training. These estimations of subject stress levels are anecdotal as no effort to formally research these levels was made. After one subject dropped out following the first week of training no further attrition occurred. Attendance at and completion of the prescribed training sessions was exceptional. Three subjects did not miss a single training session. Three subjects missed only one or two workouts due to minor illness. One subject had to cease training for one week due to more serious illness, but resumed training after that week. Two subjects attended all of the training sessions although they performed scaled down versions of two of the workouts due to minor illness. Finally, two of the subjects each missed three training sessions in a row due to work or family commitments and one of these subjects missed two other workouts due to illness or injury. However, results from these latter two subjects were not included in the final results because of these absences. Considering there were fifty-two training sessions in total, the low number of absences just described does illustrate the desire of these subjects to complete the training. If stress levels had become too high, more drop outs or greater absenteeism would have likely been observed.

When considering the perception of the training regimen as a distress or eustress, it is beneficial to consider the backgrounds of the subjects. The subjects in this study were competitive athletes. The study took place during their off-season training period. Taking part in an organized study was seen as a desirable opportunity. The study offered physiological and performance tests with which to monitor progress. Training sessions with other competitive cyclists ensured company and motivation. The fact that the training program was difficult appealed to the group because they had high competitive aspirations and goals. Although the training sessions were difficult and the subjects had to be motivated to complete the training sessions, the underlying premise was that the objective of the study was not to overtrain them. Ethical constraints eliminated overtraining as a possibility. Each subject believed that completing this training was going to help improve their performance in the upcoming competitive season. Consequently there was an underlying positive feeling about the study by the subjects. Since the total stress experienced by an individual was conditional on their perception of the stress, this generally positive feeling about the study by the majority of the subjects may have possibly had an effect on the physiological responses of the subjects.

4.2 Infectious Episodes

Susceptibility to viral infection is influenced by whether the virus is new to the organism, and whether the immune response is suppressed in some way. Several subjects did experience viral infections. One subject did become ill with the flu at the end of the volume training period. Immune cell counts while the subject was infected did not differ as compared to when the subject was healthy. Serum IL-6 concentrations in healthy subjects were undetectable, but were elevated in five subjects experiencing mild colds and when the one subject experienced the flu. These observations suggest that immune cell counts were not altered by viral infection, but serum IL-6 concentrations were. Considering that the immune cell counts were used as one indicator of immune system state, the observation of no changes in immune cell count during moderate viral infection suggests that the sensitivity of the measurement of immune cell counts to represent immune system state should be investigated further.

4.3 Future Research

As a result of this research effort, several issues regarding the study of immune function during exercise have surfaced. These issues should be investigated by future researchers.

Generally, there is a need to better understand how the immune system functions. Understanding how the components (cells and tissues) of the immune system function, how they work cooperatively with other cells and tissues, and how they affect and are affected by other systems, such as the endocrine and neural systems, is important. This section will list issues to address in future research.

4.3.1 What are the best methods to measure immune and hormone biological variables?

4.3.1.1 What are the best methods to measure immune function?

Present studies have utilized a variety of methods to assess immune function. Choosing an optimal method will be a result of understanding how the immune system works, what type of infection the subject might be susceptible to, and what biotechnical methods offer the best validity and reliability of measurement. Several possible methods are:

4.3.1.1.1

Numbers of immune cells in various body compartments can be determined. Examples are leukocyte and lymphocyte subset counts.

4.2.1.1.2

Cell activation markers can provide information about whether a cell has been activated recently. The proportion of cells with an activation marker compared to those which do not have the marker may be a useful measurement. Examples of possible markers are:

- IL-2 receptors on the surface of T cells indicate that the T cell has been activated recently.
- Parts of IL-2 receptor molecule soluble in blood is indicative of recent T cell activation.
- Lymphocyte function-associated antigen - 1 (LFA-1) is an intercellular adhesion molecule.
- Human leukocyte antigen DR allele product (HLA-DR$^+$) is an intercellular adhesion molecule important in T cell binding to antigen presenting cells and in T cell activation.

There are potentially many ways of measuring immune function (possibly more ways which are not yet determined) because there are many different cell surface molecules. As reported in a 1994 immunology text (13), there were over 109 cluster of differentiation (CD) designations.

4.3.1.1.3

Immune cell functional activity rates can be assessed by activating cells with various stimulators.

- Stimulated proliferation rates of lymphocytes to mitogens are indicators of cell activity. Common mitogens used have been concanavalin (Con-A) and phytohemagglutinin (PHA).
- Granulocyte oxidative capacity is a measurement of granulocyte activity. A common stimulus used is phorbol myristate acetate (PMA).

4.3.1.1.4

There are many messenger molecules, such as cytokines or growth factors, that act between cells of the immune, endocrine, and neural systems to coordinate immune responses. For example, as of 1994, there were 13 interleukin (IL) molecules (13).

4.3.1.1.5

The actual presence of these cells, CD markers, or molecules can be assayed; however, measuring the rate of genetic transcription of these variables may be useful.

4.3.1.2 What are the best methods to measure hormone function?

Hormones can be measured for their presence in blood or for their metabolites excreted in urine. There will not be a large discussion on this topic here except to mention that since the completion of this study, a precise mechanism of how glucocorticoids affect immune function has been published (14, 15). These studies reported that glucocorticoids inhibited the launching and proliferation of an immune response by inhibiting the translation of transcription factors for cytokines responsible for the immune response. An immune system faced with an immunological challenge may be limited in its ability to mount an appropriate response due to exercise-induced, elevated systemic glucocorticoid production. Thus, athletes may succumb to viral and bacterial infections during very stressful glucocorticoid-producing exercise challenges. Please refer to Appendix E for more information on this topic.

There were some methodological issues that were controlled for in this study and should be controlled for in future research. Differences in training, testing, and assay protocols have made comparison of results among studies difficult. In this study the training regimen was strictly controlled. All of the subjects underwent the same training protocol. The training stimulus was meant to be heavy overload endurance training, not an excessive load that would cause overtraining. Adequate rest after exercise (24 - 36 hours) and before sample collection was ensured. Urinary (24 hour) free cortisol concentration was used in order to provide a means of assessing daily cortisol production while avoiding the complications of cortisol's circadian variation, pulsatile release pattern, response to short term stress, and changes in production, binding, or clearance (16). No limit was put on other factors, such as diet or sleep, that would impose additional stresses on the subjects.

4.3.2 What do these immune measurements mean in terms of the "strength" of the immune system?

With the multiple measurement choices available, the decision to use one measurement over another is based on the knowledge of what physiological factor the measurement technique is measuring, and what a change in the measurement value potentially means in terms of subject health. How immune cells function within the whole of the organism, must be understood before making assumptions about immune capabilities. As an example, it has been reported that acute exercise causes a alteration of certain lymphocyte subset counts in blood (4, 5, 6). It has been speculated that the fall in circulating lymphocyte numbers following exercise may result in a period of increased susceptibility to infection (17). If this speculation is true, what relevance does this change in circulating lymphocyte count have for immune function? By how much must the number of lymphocytes fall in order for immune function to be suppressed? When it is understood how such changes in lymphocyte subset counts occur, hypotheses on the effects of changes in lymphocyte cell count on immune function can be formed. In order to more fully appreciate these questions it may be useful to consider a short summary on alterations of lymphocyte subset cell counts during acute endurance exercise, and the magnitude of numerical change.

4.3.2.1 Acute Exercise Lymphocyte Redistribution.

Acute exercise has been reported to bring about dramatic changes in circulating immune cell counts (4, 5, 6, 7). Exercise-induced increases in the velocity of circulating blood may physically brush immune cells off the walls of blood vessels. Changes in cell-surface receptor characteristics, which cause alterations in cell adhesion characteristics, may release cells bound to tissues into the circulation (18, 19). Epinephrine, produced during exercise, has been suggested to be a factor in altering cell-surface receptor adhesion properties (20). Circulating immune cell counts returned to normal within 24 hours following a strenuous acute exercise bout (6), supporting the possibility that the change in circulating cell counts was a result of a redistribution of cells that returns to base levels following a period of recovery. A question to ask is whether this redistribution of immune cells affects immunocompetence. . Lymphocyte recirculation (see Appendix C) refers to the movement of lymphocytes through the blood and lymphatic systems. A thorough understanding of lymphocyte redistribution, recirculation, and how the immune system responds to the introduction of a new antigen, is necessary to assess how acute exercise-induced changes in lymphocyte counts affect immune function.

4.3.2.2 The Magnitude of Change in Leukocyte Counts in Acute Exercise.

The following discussion will present examples of the percent change of certain leukocytes during acute exercise. This topic is presented to acknowledge the magnitude of change in cell counts and to question if that amount of change affects immune function. The cells most affected by an acute exercise-induced redistribution of cells are granulocytes (4, 5, 6) and natural killer cells (4, 5). T lymphocytes are affected to a lesser degree (4) and B lymphocytes are generally thought not to be greatly affected (4). Circulating concentrations of neutrophils, which make up approximately 94% of all granulocytes (13), have been reported to increase by as much as 300% after a strenuous acute bout of exercise (6). Considering that only approximately 3% of neutrophils in the human body are regularly circulating in the blood at one time (12), it should be questioned whether a three fold increase in circulating neutrophils has a significant effect on immunity. Similarly, when only 0.2% of the total lymphocyte mass circulates at one time, how does, for example, a 43% increase (5) during exercise followed by a 78% decrease (5) during recovery in circulating $T_{cytolytic}$ cell count affect immunity?

Putting in context how circulating cell counts are altered and by how much may be a primary step in understanding what a change in immune cell count means in terms of the strength of an immune response. Some questions that should be addressed in future research follow now.

- If there has been no loss of cells, as demonstrated by the fact that cell counts return to normal within 24 hours following exercise, does such an apparently small change in cell redistribution affect immunity?
- What relevance does a slight change in lymphocyte number, in a small sample of blood, have on immunity when antigen recognition generally occurs in lymph nodes or at the antigen site of entry?
- Is the number of circulating cells always directly correlated to the total number of immune cells in the body?
- If there is a fall in circulating lymphocyte count, how quickly can new lymphocytes be generated?

4.3.3 In What Conditions are these Measurements Altered?

Determining the type of infections athletes are most susceptible to, for example, upper-respiratory tract infections, will focus the choice of measurements on the aspects of immunity involved in combating that type of infection. Decisions could be made to research certain types of cells, in particular locations in the body. Relevant messenger molecules, cell-surface receptors, and/or cytokines used to activate pertinent immune cells could be measured. Thus, immune function that may be most likely affected in endurance training athletes could be monitored.

Researching the reported clinical changes in immune cell counts in normal and pathological conditions could aid in understanding how immune cell counts change during an immune challenge.

Chronic heavy training does impose a variety of metabolic stressors on the immune system such as metabolic acidosis from lactic acid production, continual fluctuation in hormone values such as epinephrine, norepinephrine, and adrenal hormones, hyperthermia, dehydration, osmotic and plasma electrolyte balance (21). It may be important to investigate how these factors affect immune function.

In summary, targeting immune function measurements towards those most likely altered in endurance athletes, researching reported changes in common infections in this category of individual, and correlating changes in immune function measurements to specific endurance exercise-induced alterations in metabolic state may be a means of approaching the question of the effects of endurance exercise and training on immune function.

4.4 Conclusion

A positive conclusion from this study is that the subjects were able to train at a high level of intensity, duration, and frequency without experiencing any alterations in the immune and hormonal variables measured. It is concluded that the type of training performed by our subjects did not increase their risk of overtraining or immunosuppression. Present results suggest that immune status as measured by whole blood lymphocyte subsets and serum cytokine concentrations of healthy cyclists, is robust and able to tolerate heavy endurance training with no apparent adverse effects. Possible reasons why no significant changes were seen in our immune and hormonal analyses were that our subjects did not train hard enough for changes to be seen, that resting lymphocyte subset counts and concentrations of cytokines are not the most appropriate indicators of the effect of training on immune status, and that measurements of immunological markers at rest, without the presence of an immunological challenge, may not reflect the functional state of the immune system.

In summary, resting lymphocyte subset counts, IL-1β, TNFα, IL-6, 24 hour urinary cortisol excretion, and serum testosterone concentration were monitored in nine male cyclists over a nine week period of heavy overload training and ten days of unloading. The data indicate that training improved aerobic performance and cycling performance without significantly altering any of the monitored immune and hormonal factors.

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Appendix A

Blood collection procedure

1. All blood samples were obtained between 7:00 and 8:30 a.m. If subjects were not able to attend between these times, samples were obtained between 8:30 and 9:30 a.m. This happened on two occasions.
2. Individual subjects had their blood sample taken at a consistent time, within 30 minutes, between 7:00 to 8:00 a.m.. All blood samples were taken while the subject was sitting down.
3. Subjects were asked to maintain a similar sequence of events each morning prior to blood collection. It was up to each subject whether they ate a meal before arriving for their sample collection.
4. Samples were obtained in a laboratory in the Department of Physical Education and Recreation at the University of Alberta.
5. Leukocyte and Lymphocyte subset measurements:

   Samples were obtained in a mauve top EDTA 5 ml vacutainer. The samples were placed in a container meeting legal safety requirements for transporting samples of human body fluids. The samples were walked across campus, a distance of approximately 1.0 km and taking ten minutes to complete, to a lab in the Department of Laboratory Medicine and Pathology. It was unlikely that the temperature of the samples changed. Precautions were taken to walk in doors during the winter months.

6. Cytokine and testosterone measurements:

Whole blood was collected in a 10 ml red-top vacutainer. The sample was allowed to clot, sitting at room temperature for approximately 30 minutes while exposed to room air. The resultant serum was poured out of the vacutainer into microcentrifuge tubes for storage. Samples were stored at -80° Celcius until determinations of serum cytokine and testosterone concentrations were commenced.

Appendix B

Determination of Ventilatory Threshold (T_{vent})

B - 1 Brief Review of the Physiology of T_{vent}

B - 1.1 Rise in $\dot{V}_E/\dot{V}O_2$ Without a Rise in $\dot{V}_E/\dot{V}CO_2$.

Wasserman et al. (1) considered the "anaerobic threshold" to be the oxygen consumption ($\dot{V}O_2$) at which anaerobic supplementation of aerobic energy production occurs. An increased anaerobic energy production results in a net increase in blood lactate. The accumulating lactic acid is immediately buffered predominantly by bicarbonate (HCO_3^-), generating additional carbon dioxide (CO_2) (1). Ventilation ($\dot{V}_E$) is increased in an attempt to exhale the additional CO_2 (2). This results in a disproportionate rise in $\dot{V}_E$ in relation to $\dot{V}O_2$ and consequently, the ventilatory equivalent of oxygen ($\dot{V}_E/\dot{V}O_2$) increases. The combined effect of a higher $\dot{V}_E$ and $\dot{V}CO_2$ results in these two variables increasing proportionately to one another (2, 3), resulting in no systematic alteration, or rise, in the ventilatory equivalent of carbon dioxide ($\dot{V}_E/\dot{V}CO_2$). A systematic increase in $\dot{V}_E/\dot{V}O_2$ without a concomitant increase in $\dot{V}_E/\dot{V}CO_2$ has been termed the "anaerobic threshold" by Wasserman et al. (2) and Davis et al. (4), and the "aerobic threshold" by Skinner and McLellan (3).

In the early stages of progressive exercise, $\dot{V}_E/\dot{V}O_2$ and $\dot{V}_E/\dot{V}CO_2$ decreases because the ratio of physiological dead space (V_D) to tidal volume (V_T) decreases (5). A proportionately larger increase in V_T to a smaller increase in V_D explains the decrease in the V_D / V_T ratio (6). The decrease in the V_D / V_T ratio becomes less steep as the work rate continues to increase (5). Therefore, the $\dot{V}_E/\dot{V}O_2$ and $\dot{V}_E/\dot{V}CO_2$ ratios decrease until additional CO_2 is exhaled, causing both $\dot{V}_E$ and $\dot{V}CO_2$ to increase. The result is that $\dot{V}_E/\dot{V}O_2$ starts to increase. Bhambhani and Singh (7) suggested that the power output at which the $\dot{V}_E/\dot{V}O_2$ ratio reaches a minimum be called "ventilatory threshold one". "Ventilatory threshold one" corresponds to the term "aerobic threshold" suggested by Skinner and McLellan (3) and "anaerobic threshold" by Wasserman et al. (2). The percentage of $\dot{V}O_{2max}$ at which ventilatory threshold I occurred at was reported to be 45.1 ± 6.9 % (7) and between 40 and 60 % (3).

B - 1.2 Non-linear Rise in $\dot{V}_E/\dot{V}CO_2$.

As the power output increases further, $\dot{V}_E$ starts to increase more rapidly than $\dot{V}CO_2$ and consequently $\dot{V}_E/\dot{V}CO_2$ starts to rise. The point at which bicarbonate buffering of lactic acid causes a parallel increase in $\dot{V}_E$ and $\dot{V}CO_2$, resulting in stable $\dot{V}_E/\dot{V}CO_2$ values, has been termed the period of "isocapnic buffering" (2). The period of marked hyperventilation following the period of isocapnic buffering has been referred to as "ventilatory or respiratory compensation" (1). This has been explained by arterial chemoreceptors detecting the increased hydrogen ion concentration, produced by the dissociation of lactic acid into lactate and hydrogen ion, stimulating an increased ventilatory drive (1), and decreasing the partial pressure of arterial CO_2 (P_aCO_2). Skinner and McLellan (3) referred to the point that $\dot{V}_E/\dot{V}CO_2$ increases non-linearly as the "anaerobic threshold". Bhambhani and Singh (7) suggested that the power output at which $\dot{V}_E/\dot{V}CO_2$ reaches a minimum be termed "ventilatory threshold two". This second threshold has been reported to occur at 68.8 ± 11.0 % (7) and between 65 - 90 % (3) of $\dot{V}O_{2max}$ when measured as $\dot{V}O_2$.

B - 2 Determination of T_{vent} For the Purpose of This Study.

In the present study, ventilatory threshold (T_{vent}) was chosen as the power output just below a non-linear or distinct rise in $\dot{V}_E/\dot{V}CO_2$. This distinct rise in $\dot{V}_E/\dot{V}CO_2$ could have been considered "anaerobic threshold" (3) or, alternatively, the point at which isocapnic buffering ended and respiratory compensation began, following "anaerobic threshold" (1). T_{vent} could have also been considered similar to "ventilatory threshold two", suggested by Bhambhani and Singh (7), although T_{vent} rarely occurred at the power output which $\dot{V}_E/\dot{V}CO_2$ was a minimum. Typically, $\dot{V}_E/\dot{V}CO_2$ was rising slightly before it increased non-linearly or "distinctly" at T_{vent}.

Figure B-1 is a typical example of a $T_{vent}/\dot{V}O_{2max}$ test result for one subject. At point I, $\dot{V}_E/\dot{V}O_2$ started to increase while $\dot{V}_E/\dot{V}CO_2$ did not increase. This point I corresponded to a $\dot{V}O_2$ of 59.6 % of $\dot{V}O_{2max}$ for this subject. At point II, both $\dot{V}_E/\dot{V}CO_2$ and $\dot{V}_E/\dot{V}O_2$ distinctly increased. $\dot{V}O_2$ at point II was 86.1 % of $\dot{V}O_{2max}$ for this subject. The power output just prior to II was considered T_{vent}. The power output just prior to point II was reported as power output at T_{vent} (POT_{vent}). The $\dot{V}O_2$ at the power output just prior to the marked increase in $\dot{V}_E/\dot{V}CO_2$ was considered $\dot{V}O_2$ at T_{vent}. The mean (± SD) percentage of $\dot{V}O_{2max}$ that T_{vent} occurred at, across the four study phases, ranged from 72 (± 8.5) to 78 (± 8.5) %. The heart rate at T_{vent}, as a percentage of HR_{max} ranged from means (± SD) of 91 (± 1.7) to 93 (± 1.9) %. These percentages are similar to those reported in a study by Ribeiro et al. (8) where mean heart rate at anaerobic threshold (0.97) was 93 ± 3 % (range 87 - 98 %) of the peak heart rate attained in a maximal incremental cycle ergometer test.

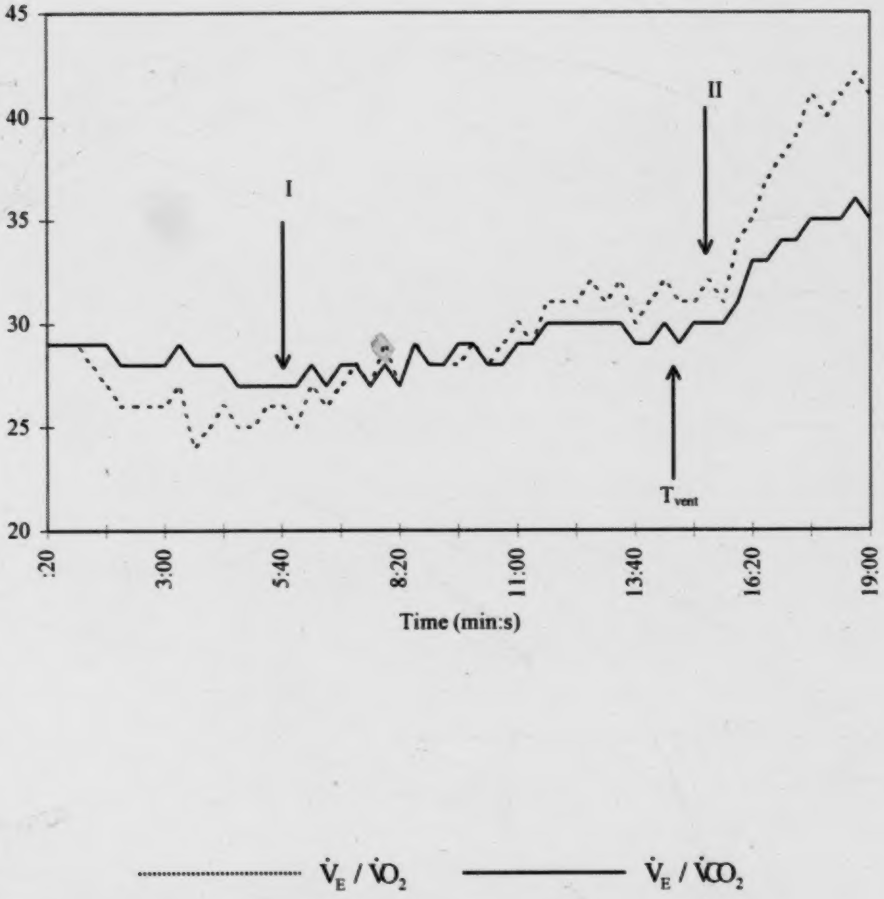

Figure B - 1. Position of ventilatory threshold during a ventilatory threshold (T_{vent}) / maximal oxygen consumption ($\dot{V}O_{2max}$) test for subject #9 at test I.

B - 3 References

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Appendix C

General Overview of the Immune System

The information for this general overview of the immune system was obtained from the textbook **Cellular and Molecular Immunology**, 2nd edition, written by A.K. Abbas, A.H. Lichtman, and J.S. Pober., Philadelphia, PA: W.B. Saunders Company.

C - 1 Introduction

The immune system is divided into two functional categories which are natural and specific. The components of natural (or innate) immunity are present in an organism prior to exposure to infectious microbes or other foreign macromolecules, are not enhanced by successive exposures to foreign substances, and do not discriminate among foreign substances. The components of specific (or acquired) immunity are stimulated by exposure to foreign substances, are specific for distinct macromolecules, and increase in magnitude of response and in defensive capabilities with each successive exposure to a particular macromolecule. Although these two classes of immune response are distinct from one another by definition, their cells and molecules do function together cooperatively. Figure C - 1 depicts the overall organization of the immune system.

C-2 Natural Immunity

Natural immunity is an organism's first line of defense. The components of natural immunity are already present in an organism prior to being exposed to the injurious agent, whereas in specific immunity, components are stimulated after exposure to the injurious agent. The cells and molecules of natural immunity combat the injurious agents by immediately phagocytizing it or by signaling to specific immune cells to come and help get rid of the foreign substance. Families of cells that are classified under natural immunity are mononuclear phagocytes, dendritic cells, granulocytes, and natural killer cells.

C - 2.1 Mononuclear Phagocytes

The function of the cells of the mononuclear phagocyte system is, as their name suggests, phagocytosis. Macrophages are the most commonly mentioned cells of this system. Understanding the development of the cells in this system will help one understand all of the cells in this system. Please see Figure C - 2.

Originating in bone marrow, the first cell of this system is the stem cell. Still in the bone marrow, stem cells mature into monoblasts. Monoblasts, after entering the peripheral blood, mature into monocytes. Monocytes contain lysosomes and phagocytic vacuoles which later play important roles in phagocytosis. Monocytes, once settled into tissue, become macrophages. At this point, macrophages either become activated or they differentiate. Macrophages can be activated by a variety of stimuli. Cytokines are key stimulators of macrophages. The section on cytokines, at the end of this review, indicates which cytokines and growth factors are active in stimulating macrophages. Upon activation, macrophages can become activated macrophages, epithelioid cells, or giant cells. Depending upon the tissue a macrophage settles in, macrophages can differentiate into a variety of different cells. In the central nervous system, macrophages differentiate into microglia, in the liver, kupffer cells, and in the lungs, alveolar macrophages.

C - 2.2 Dendritic Cells

Dendritic cells are classified as interdigitating dendritic cells and follicular dendritic cells. The function of interdigitating dendritic cells is to present protein antigens to $CD4^+$ T_{helper} lymphocytes. For definition purposes, an antigen is a foreign substance that induces specific immunity. "Presenting" antigen refers to the action of a cell engulfing an antigen and then presenting a piece of that antigen on it's surface. This presented piece of antigen will be recognized as foreign by a T cell. Interdigitating dendritic cells are present in the interstitium of most organs, in lymph nodes and spleen, and are scattered throughout the body's epidermis. The function of follicular dendritic cells is to trap antigens complexed to antibodies or complement products and display them on their surfaces in order for B lymphocytes to recognize the antigen. Follicular dendritic cells are present in lymph nodes, spleen, and mucosa-associated lymphoid tissues

C - 2.3 Granulocytes

Granulocytes are so named because these leukocytes contain abundant cytoplasmic granules. These leukocytes play an important role in inflammation and as participants in both natural and specific immune responses. The three types of granulocytes and their relative percent contribution of the total granulocyte population are neutrophils (polymorphonuclear leukocytes) (94%), eosinophils (5%), and basophils (1%).

C - 2.4 Neutrophils

Neutrophils are the most numerous of the granulocytes. Their function is to phagocytize foreign particles. They respond quickly to chemotactic stimuli (e.g. histamine) and are activated by cytokines produced by T cells, macrophages and endothelial cells, and by complement protein. Neutrophils are the cell population that responds the most during acute phase responses.

C - 2.5 Eosinophils

Eosinophils express receptors for a particular class of antibodies called IgE. The type of infectious agents that stimulate IgE are parasites such as helminths (worms). Eosinophils are abundant at sites of immediate hypersensitivity (allergic) reactions.

C - 2.6 Basophils

The granule contents of basophils (e.g. histamine, lipid mediators such as leukotrienes and prostaglandin D_2) are the chemical mediators of immediate hypersensitivity (allergic) reactions. Basophils express high-affinity receptors for IgE. Thus, basophils are effector cells of IgE-mediated immediate hypersensitivity.

C - 2.7 Natural Killer Cells

Natural killer (NK) cells are large granular lymphocytes. Natural killer cells are not classified under specific immunity, like T and B cells, because they do not have the ability to specifically recognize different antigenic determinants (an antigenic determinant is the portion of an antigen that is recognized as foreign).

Natural killer cells are capable of lysing a variety of tumor- and virus- infected cells. Natural killer cells are similar to $T_{cytotoxic}$ cells except that they lack the specific T cell receptor for antigen recognition. Since killing ability is not induced by a specific antigen, natural killer cells are considered part of natural immunity rather than specific immunity. However, natural killer cell killing is not random. The molecular structure on the surface of susceptible target cells, that may be recognized by natural killer cells, has not yet been defined. Natural killer cells can be activated by cytokines produced by $CD4^+$ T_{helper} cells, and targets coated with IgG antibodies. This second form of cytolysis is called antibody-dependent cell-mediated cytotoxicity (ADCC).

C - 3 Specific Immunity

Specific immunity is stimulated when an organism is exposed to antigen. Antigens are defined as foreign substances that induce specific immunity. Specific immunity is also known as acquired immunity. Specific immunity is classified into two types of immune responses: humoral immunity and cell-mediated immunity.

C - 3.1 Humoral Immunity

Humoral immunity obtained it's name from the fact that proteins, called antibodies (Ab), present in body fluids (humors) mediate the immune response. Antibodies are produced exclusively by cells of the B lymphocyte lineage. The function of antibodies is to neutralize and eliminate the antigen that induced antibody formation. Another name for antibody is immunoglobulin (Ig). Production of all antibodies is initiated by the interaction of antigen with a small number of mature B cell-bound IgM or IgD (2 classes of antibody) specific for that antigen. This interaction occurs in peripheral lymphoid tissues (lymph nodes, spleen, mucosa-associated lymphoid tissue, and the cutaneous immune system). An antigen bound to membrane IgM or IgD on a specific B lymphocyte initiates a series of responses that lead to two principal changes in that B lymphocyte: proliferation and differentiation. Proliferation refers to the cloning and production of many more B lymphocytes of the same type as the original. Differentiation refers to the acquisition of more mature characteristics by B lymphocytes. Differentiation also refers to the production of memory B cells.

There are several biological effector functions mediated by antibodies. The effector function carried out depends on the structure of the antibody, the anatomic location where the antibody-antigen binding took place, and the isotype of the antibody (IgM, IgD, IgA, IgE, and IgG). Several types of biological effects are:

C - 3.1.1

Antigen bound to antibody expressed on the surface of a B lymphocyte will cause that B lymphocyte to proliferate and secrete more antibodies specific for that particular antigen.

C - 3.1.2

Secreted antibodies can sterically hinder the binding of many injurious agents (e.g. toxins, drugs, viruses, bacteria, and parasites) to specific cell surface receptors, thus blocking injury to the cell. Antibodies do this by binding to the antigenic determinant on the injurious agents, thereby neutralizing the toxic or infectious process.

C - 3.1.3

Depending on the isotype of antibody that recognizes the antigen, the method by which the antibody aids in eliminating an antigen varies. Several examples will now be discussed:

C - 3.1.3.1

The complement system is activated by IgG and IgM. The complement system is a cascading system of functionally linked proteins that mediate cytolysis of cells by forming pores in membranes or by disrupting the phospholipid bilayer of the membrane of these cells. Thus, these cells are killed by osmotic lysis. The complement system also plays a role in the opsonization of foreign organisms or particles. Opsonization refers to the action of complement proteins binding all over the surface of a foreign organism or particle. Phagocytic cells then recognize the opsonins (the complement proteins on the foreign body) and phagocytize the foreign body. Complement also plays an important role in the activation of inflammation. When the cascade of complement proteins occurs, certain proteolytic fragments of complement proteins are generated. These proteolytic fragments perform several functions. They activate mast cells, vascular endothelium, and inflammatory leukocytes, and also enhance the B lymphocyte response to antigen.

C - 3.1.3.2

Phagocytosis is enhanced by opsonization by IgG. Similar to how complement system proteins enhance phagocytosis by opsonizing the foreign organism or particle, opsonization by IgG can enhance phagocytosis.

C - 3.1.3.3

Antibody-dependent cell-mediated cytotoxicity (ADCC) is when a foreign cell is coated with a specific antibody (particularly IgG, IgE, and IgA) and is thus recognized by one of the several different leukocytes: neutrophils, eosinophils, mononuclear phagocytes, and natural killer cells. The result is that the coated foreign cell is lysed or phagocytized.

C - 3.1.3.4

Mast cells and basophils express high-affinity receptors for IgE antibodies.

C - 3.1.3.5

IgA plays an important role in mucosal immunity. IgA is the only antibody that can be selectively transported across mucosal barriers. IgA is synthesized in mucosal lymphoid tissues and it functions to neutralize injurious agents in mucosal secretions.

C - 3.1.3.6

Maternally produced antibodies can provide protection for the fetus or baby. Maternal IgG is transported across the placenta and enters the fetal circulation. In addition, maternal IgA and IgG are secreted into breast milk.

C - 3.2 Cell-mediated Immunity

T lymphocytes are the effector cells of cell-mediated immunity. There are two classes of T lymphocytes that are functionally distinct from one another. These are $CD4^+$ T_{helper} cells and $CD8^+$ $T_{cytolytic}$ cells. "CD" is short form for "cluster of differentiation". A cluster of differentiation is a marker on the surface of a cell which has a structure that defines a particular cell lineage or growth stage. To recognize this marker, monoclonal antibodies are used. The technique of flow cytometry is often used to determine counts of cells expressing different cell surface markers.

T lymphocytes have receptors on their outer membranes that recognize and bind antigens. T lymphocytes only recognize peptide antigens that are attached to proteins which are encoded by the major histocompatability complex (MHC). The MHC is a highly variable region of genes whose products are expressed on the surfaces of a variety of cells. T lymphocytes do not recognize antigens in free or soluble form, but do recognize portions of protein antigens (i.e. peptides) that are non-covalently bound to MHC gene products. Therefore, MHC molecules display antigenic peptides to T cells. T cells can then survey the body for foreign proteins that are presented by MHC molecules. There are two different types of MHC gene product: class I and class II MHC molecules. Any particular T cell recognizes either class I or class II MHC bound antigen but not both. T cells that recognize class I MHC are CD8$^+$ $T_{cytolytic}$ cells. T cells that recognize class II MHC are CD4$^+$ T_{helper} cells. Types of cells that present antigen bound to either class I or class II MHC are called antigen-presenting cells (APCs). The type of APC for CD4$^+$ T_{helper} cells differs from that for CD8$^+$ $T_{cytolytic}$ cells. For CD4$^+$ T_{helper} cells to recognize antigen presented on an APC, there are two requisite properties that the APC must have. The APC must be able to process endocytosed antigens, and express class II MHC gene products. Such cells include mononuclear phagocytes, B lymphocytes, dendritic cells, Langerhans cells of the skin, and endothelial cells. Not many of these cells actually continuously express class II molecules on their surface. It often takes CD4$^+$ T_{helper} cell derived cytokines to signal these cells to express class II MHC. In contrast to this, almost all cells express class I MHC. CD8$^+$ $T_{cytolytic}$ cells recognize class I MHC molecules. $T_{cytolytic}$ cells are the primary immunological defense against viruses and tumor cells. The expression of class I MHC molecules on almost all cells ensures that any cell synthesizing viral or mutant proteins can be marked for recognition and killing by CD8$^+$ T$_{cytolytic}$ cells.

C - 3.2.1 Effector Mechanisms:

C - 3.2.1.1 CD4$^+$ T$_{helper}$ Lymphocytes

CD4$^+$ T$_{helper}$ cells initiate specific immunity, both cell-mediated and humoral, by recognizing portions of protein antigens bound to class II MHC molecules on the surface of APCs. By means of cytokine secretion, CD4$^+$ T$_{helper}$ cells stimulate the function of and focus the activity of nonspecific effector cells of natural immunity, thereby converting these cells into agents of specific immunity. For example, CD4$^+$ T$_{helper}$ cells secrete the cytokine interleukin 2 (IL-2), which in turn activates natural killer, T, and B lymphocytes. Interferon-gamma (IFN-γ) activates mononuclear phagocytes, and tumor necrosis factor (TNF) and lymphotoxin (LT) activate neutrophils and endothelial cells. There is evidence that CD4$^+$ T$_{helper}$ cells differentiate into subsets that produce distinct patterns of cytokine production and thus perform distinct functions. For example, there is thought to be CD4$^+$ T$_H$1 (T$_{helper}$ 1) cells and CD4$^+$ T$_H$2 (T$_{helper}$ 2) cells. T$_H$1 cells produce INF-γ, TNF, LT, and IL-2. T$_H$2 cells produce IL-4, IL-5, and IL-10. T$_H$1 cells participate in an effector mechanism called Delayed Type Hypersensitivity (DTH) and T$_H$2 cells participate in immediate hypersensitivity, or allergic reactions. The DTH reaction will be discussed now. The ultimate effector cell in the DTH reaction is the activated mononuclear phagocyte (macrophage). The DTH reaction process is as follows: (1) CD4$^+$ T$_H$1 cells recognize foreign protein antigens presented on the surface of APCs, (2) CD4$^+$ T$_H$1 cells secrete cytokines and proliferate, (3) two possible effector phases are (a) inflammation (vascular endothelial cells recruit circulating leukocytes into the tissues at the local site of antigen challenge), and (b) resolution (macrophages attempt to eliminate the antigen).

C - 3.2.1.2 CD8$^+$ T$_{cytolytic}$ Lymphocytes

CD8$^+$ T$_{cytolytic}$ cells kill target cells by lysing them. The CD8$^+$ T$_{cytolytic}$ cell first has to recognize an antigen presented by a class I MHC on an APC. Cell contact between the CD8$^+$ T$_{cytolytic}$ cell and target cell is required in order for the CD8$^+$ T$_{cytolytic}$ cell to carry out it's "lethal hit". A membrane pore-forming protein called perforin, and an apoptosis causing-enzyme are secreted from the CD8$^+$ T$_{cytolytic}$ cell and deposited into the target cell. The CD8$^+$ T$_{cytolytic}$ cell detaches from the target cell and the target cell then undergoes apoptosis and osmotic lysis. Apoptosis refers to the killing of a target cell by the fragmentation of the target cell's DNA. CD8$^+$ T$_{cytolytic}$ cells also transcribe and secrete cytokines (eg. IFN-γ, LT, and TNF) and other proteins upon activation. As a result of the production of a range of cytokines, CD8$^+$ T$_{cytolytic}$ cells are also stimulators of natural immunity.

C - 4 Cytokines

Cytokines are protein hormones that mediate the effector phases of both natural and specific immunity. Technically speaking the cytokines that mediate natural immunity, and are produced by mononuclear phagocytes, are called monokines and those that mediate specific immunity and are thus secreted by lymphocytes are called lymphokines. Cytokines that stimulate the growth and differentiation of immature leukocytes in the bone marrow are called colony-stimulating factors (CSFs).

Cytokines have numerous functions. Tables C - 1 to C - 4 give an overview of the functions of many cytokines. New cytokines are still being discovered, so this can not be considered a complete list.

C - 4.1 Mediators of Natural Immunity.

These cytokines protect against viral infection and initiate inflammatory reactions that protect against bacteria. They are mainly produced by mononuclear phagocytes, although many are produced by T cells in order that both specific and natural immune systems function cooperatively. See Table C - 1.

C - 4.2 Mediators of Specific Immune Responses.

C - 4.2.1 Mediators of Growth and Differentiation of Lymphocytes.

These cytokines activate both cell-mediated and humoral immune responses. Most of these cytokines are produced by $CD4^+$ T_{helper} lymphocytes. See Table C - 2.

C - 4.2.2 Mediators of Cell-mediated Immune Responses.

The cell that produces and secretes these cytokines is mainly the T cell, with the exception of IFN-γ which is also secreted by natural killer cells, and IL-12 which is only produced by macrophages. See Table C - 3.

C - 4.3 Mediators of Growth and Differentiation

See Table C - 4.

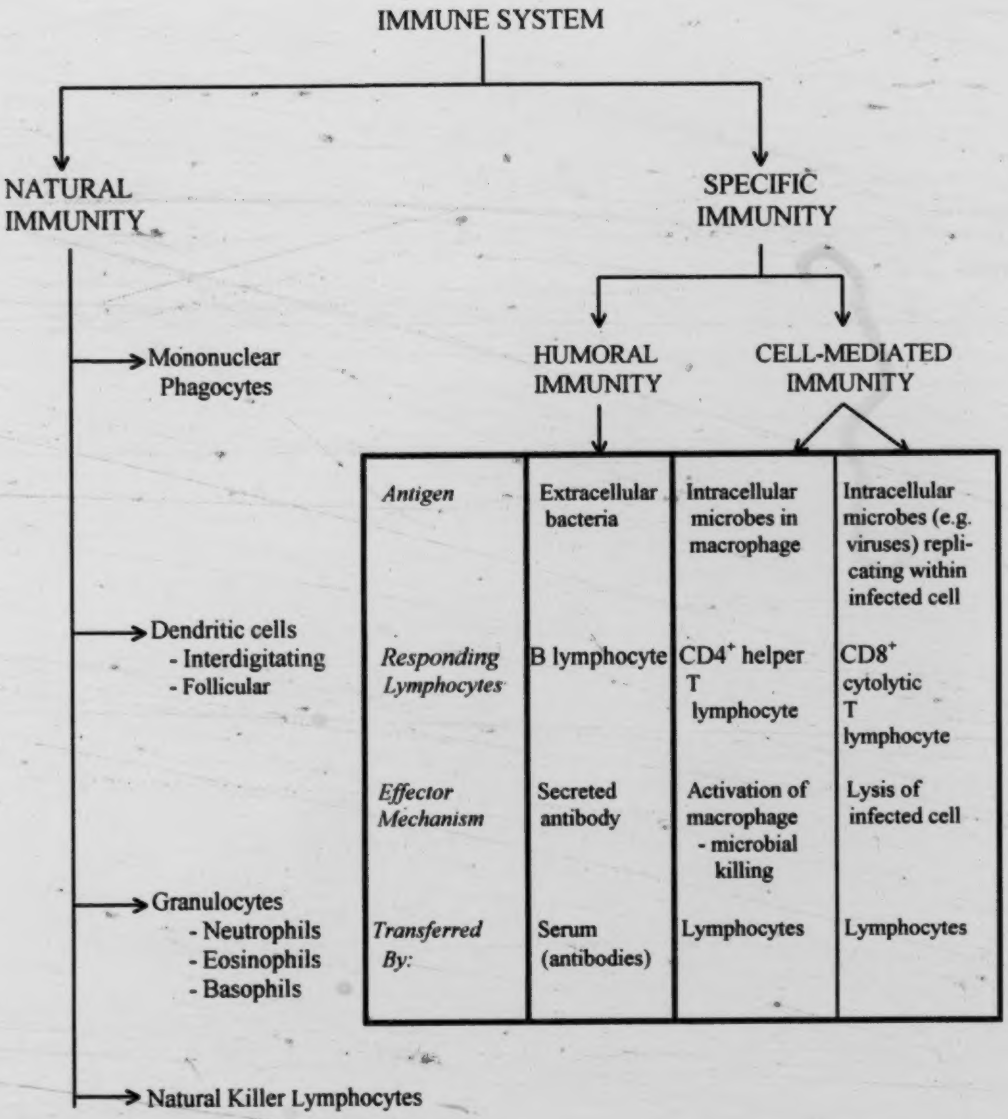
C - 5 Immune Cell Circulation

This topic will not be described in great detail here. Please consult an immunology textbook for more information.

Immune system cells and organs launch defenses against invading foreign pathogens that enter the body. The most common sites of entry are through the mucosa-lined gastrointestinal and respiratory tracts, and through the cutaneous organ or "skin". Many of the immune system cells can be found in these physical areas of the body. The lymphatic system is a system comprised of vessels and ducts, lymph nodes, and spleen. This system collects foreign pathogens from their location of entry and transports them to lymph nodes, where many lymphocytes can become in contact with the pathogen.

Depending upon the anatomic location within an organism, the type and function of an immune cell can vary. Some cells are specialized for certain tissues, such as keratinocytes which are found in epithelial cells of the skin, whereas other cells circulate between tissues. Most lymphocytes circulate through the organism. This phenomenon is called "lymphocyte recirculation". Lymphocyte recirculation is a continuous, non-random migration of lymphocytes from the blood and lymphatic systems to lymphoid tissues and back to the blood steam. The purpose of this recirculation is to ensure that a large number of lymphocytes representing diverse clones can be circulated through locations where foreign antigens are localized (lymph nodes). Considering that any one antigen can be recognized by only one in 10^6 T or B cells, this method of circulating lymphocytes past new foreign antigens is necessary. Cell-surface adhesion molecules on lymphocytes control the migration of these cells through the recirculation pattern. It is interesting to note that only 0.2% of the total lymphocyte mass circulates in the blood at one time.

Understanding the anatomic physiology of the immune system is as important as understanding the functions of the individual cells themselves.


IMMUNE SYSTEM
NATURAL IMMUNITY
SPECIFIC IMMUNITY
HUMORAL IMMUNITY
CELL-MEDIATED IMMUNITY
Mononuclear Phagocytes
Dendritic cells
- Interdigitating
- Follicular
Granulocytes
- Neutrophils
- Eosinophils
- Basophils
Natural Killer Lymphocytes

Antigen	Extracellular bacteria	Intracellular microbes in macrophage	Intracellular microbes (e.g. viruses) replicating within infected cell
Responding Lymphocytes	B lymphocyte	$CD4^+$ helper T lymphocyte	$CD8^+$ cytolytic T lymphocyte
Effector Mechanism	Secreted antibody	Activation of macrophage - microbial killing	Lysis of infected cell
Transferred By:	Serum (antibodies)	Lymphocytes	Lymphocytes

Figure C - 1. Overall organization of the immune system. The information for this figure was obtained from Abas, A.K., Lichtman, A.H, and J.S. Pober (1994) **Cellular and Molecular Immunology**, 2nd Edition. Philadelphia, PA: W.B. Saunders Company.

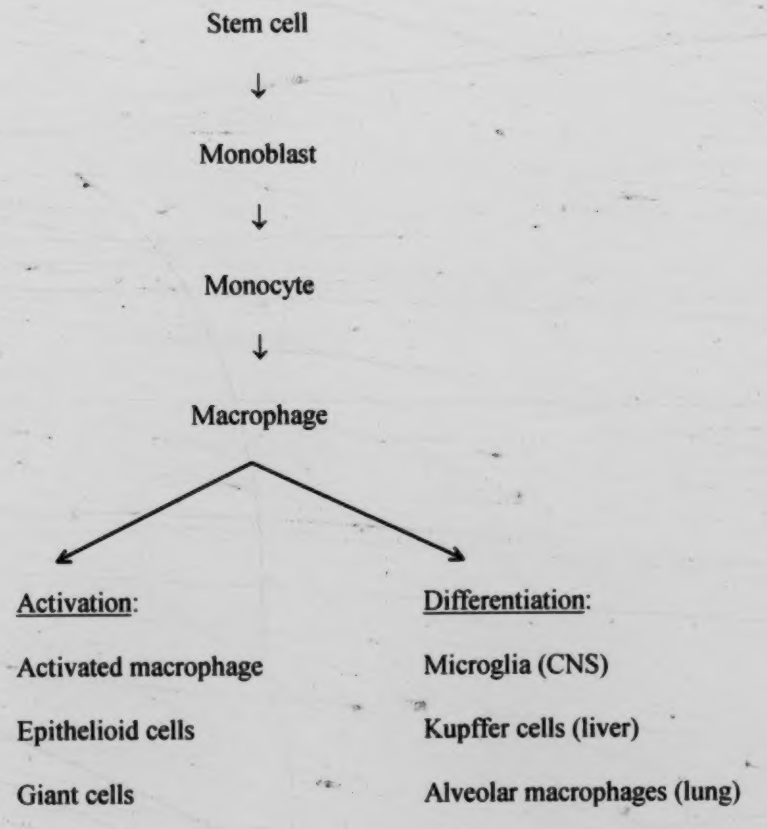

Stem cell
Monoblast
Monocyte
Macrophage
Activation:
Activated macrophage
Epithelioid cells
Giant cells
Differentiation:
Microglia (CNS)
Kupffer cells (liver)
Alveolar macrophages (lung)

Figure C - 2. Mononuclear phagocyte growth pattern. The information for this figure was obtained from Figure 2-5 in Abbas, A.K. Licktman, A.H., and H.S. Pober. (1994) **Cellular and Molecular Immunology**. 2nd Edition. Philadelphia, PA: W.B. Saunders Company.

Table C - 1. Mediators of Natural Immunity. The information for this table was obtained from Table 12-1 of Abbas, A.K., Lichtman, A.H., and J.S. Pober (1994) **Cellular and Molecular Endocrinology**. Philadelphia, PA: W.B. Saunders Company.

Cytokine	Cell Target	Primary Effects on Each Target
Type I IFN	All cells	- Antiviral, antiproliferative, increased class I MHC expression
	NK cells	- Activation
Tumor Necrosis Factor (TNF)	Neutrophils	- Activation (inflammation)
	Endothelialcells	- Activation (inflammation, coagulation)
	Hypothalamus	- Fever
	Liver	- Acute phase reactants (serum amyloid A protein)
	Muscle, fat	- Catabolism (cachexia)
	Thymocyte	- Costimulator
Interleukin-1 (IL-1)	Thymocyte	- Costimulator
	Endothelial cell	- Activation (inflammation, coagulation)
	Hypothalamus	- Fever
	Liver	- Acute phase reactants (serum amyloid A protein)
	Muscle, fat	- Catabolism (cachexia)
Interleukin-6 (IL-6)	Thymocyte	- Costimulator
	Mature B cell	- Growth
	Liver	- Acute phase reactants (fibrinogen)
Chemokines	Leukocytes	- Leukocyte chemotaxis and activation

Table C - 2. Mediators of Specific Immunity. Mediators of Growth and Differentiation of Lymphocytes. The information for this table was obtained from Table 12-2 of Abbas, A.K., Lichtman, A.H., and J.S. Pober (1994) **Cellular and Molecular Endocrinology**. Philadelphia, PA: W.B. Saunders Company.

Cytokine	Cell Target	Primary Effects on Each Target
Interleukin-2 (IL-2)	T cell	- Growth; cytokine production
	NK cell	- Growth, activation
	B cell	- Growth, antibody synthesis
Interleukin-4 (IL-4)	B cell	- Isotype switching to IgE
	Mononuclear phagocyte	- Inhibit activation
	T cell	- Growth
Transforming growth factor-β	T cell	- Inhibit activation and proliferation
	Mononuclear phagocyte	- Inhibit activation
	Other cell types	- Growth regulation

Table C - 3. Mediators of Specific Immunity. Mediators of Cell-mediated Immune Responses. The information for this table was obtained from Table 12-3 of Abbas, A.K., Lichtman, A.H., and J.S. (1994) **Cellular and Molecular Endocrinology**. Philadelphia, PA: W.B. Saunders Company.

Cytokine	Cell Target	Primary Effects on Each Target
Gamma Interferon (γIFN)	Mononuclear phagocyte Endothelial cell NK cell All	- Activation - Activation - Activation - Increased class I and class II MHC molecules
Lymphotoxin	Neutrophil Endothelial cell NK cell	- Activation - Activation - Activation
Interleukin-10 (IL-10)	Mononuclear phagocyte B cell	- Inhibition - Activation
Interleukin-5 (IL-5)	Eosinophil B cell	- Activation - Growth and activation
Interleukin-12 (IL-12)	NK cells T cells	- Activation - Activation (growth and differentiation)
Migration inhibition factor	Mononuclear phagocyte	- Conversion from motile to immotile state

Table C - 4. Mediators of Growth and Differentiation. The information for this table was obtained from Table 12-4 of Abbas, A.K., Lichtman, A.H., and J.S. Pober (1994) **Cellular and Molecular Endocrinology**. Philadelphia, PA: W.B. Saunders Company.

Cytokine	Cell Target	Primary Effects on Each Target
c-Kit ligand	Pluripotent stem cell	- Activation
Interleukin-3 (IL-3)	Immature progenitor	- Growth and differentiation to all cell lines
Granulocyte-macrophage CSF	Immature progenitor	- Growth and differentiation to all cell lines
	Committed progenitor	- Differentiation to granulocytes and mononuclear phagocytes
	Mononuclear phagocyte	- Activation
Macrophage CSF	Committed progenitor	- Differentiation to mononuclear phagocytes
Granulocyte CSF	Committed progenitor	- Differentiation to granulocytes
Interleukin-7 (IL-7)	Immature progenitor	- Growth and differentiation to B lymphocytes

Appendix D

Overview of the Technique of Flow Cytometry

The leukocyte counts and lymphocyte subset counts in this study were determined by immunophenotyping using dual fluorescence flow cytometry. Immunophenotyping refers to the technique of identifying cells by using antibodies (Ab) that are specific for, and thus bind to, antigens expressed on the surface of the cells in question (1). "Flow cytometry is a process in which individual cells, or other biological properties, are made to pass in single file, in a fluid stream, by a sensor or sensors which measure physical or chemical characteristics of the cells or particles" (2). The characteristics that were measured in this study were cell surface receptors (i.e., markers) that are known to be present on particular mature leukocyte lineages.

This overview will start with a description of flow cytometry, and in particular dual fluorescence flow cytometry. It will then conclude with a discussion of how immunological analysis using fluorescent labeled antibodies was carried out.

D - 1 Flow Cytometry

This section will briefly explain how a flow cytometer works. A key reference for this section is the text Practical Flow Cytometry by Shapiro (2). Please refer to this text for a diagram of a typical flow cytometer and specific diagrams of a flow cytometer light source, collector, and detection system.

Flow cytometers measure fluorescence emission from, and light scatter by, each cell or particle that passes through it. The pattern of light emission and scatter is detected, recorded, and displayed for the user of the system. The function of each part of a flow cytometer will be described in the following sections.

D - 2 The Flow System

Sample cells, in a suspension, are coaxially (parallel) injected into a much more rapidly moving column or "sheath" of fluid. A 25 to 29 gauge needle is used as the injector. This rapidly moving sheath of fluid causes the alignment of the injected cells to be a single file line within the sheath. The flow velocities of the sheath and injected cells are adjusted so that the flow is laminar, thus confining the injected cell-filled fluid to the central region of the stream. Typically, the moving fluid sheath flows at a velocity of nearly 10,000 times faster than the injected cells (3). This stream of cells and fluid sheath are directed down past a precisely focused light beam. The intersection of the sample stream and beam of light is called the "observation point", and is where the measurement of the sample cells or particles takes place. Many different biological particles, including cells, immune complexes, liposomes, bacteria, fungi, and chromosomes can be measured by flow cytometry. Since this study measured the presence of particular cell types, this overview will refer to the substances being sampled as cells. It should be kept in mind, though, that cells are not the only substances that can be measured by flow cytometry.

Requirements of the observation point, in order to make measurements of the sample, are that ① the fluid stream has to be illuminated with a light source, and ② the fluorescence emission and scattered light have to be collected and directed towards a detector in order to be measured.

D - 2.1 Light Source

A common light source used, and the light source used in this study, is a laser (Light Amplified Stimulated Emitted Radiation). An important characteristic of the light source used in flow cytometry is brightness; and brighter is better. Laser light is monochromatic; that is, the photons making up the light are traveling parallel to one another at the same wavelength. The result is that bright light can be focused on a small area such as the observation point. The most popular laser used for flow cytometry, and that used for this study, is an argon ion laser. The argon ion laser runs at a wavelength of 488 nm which is ideal for exciting the fluorescent markers fluorescein isothiocyanate (FITC) and phycoerythrin (PE). The excitation wavelength for FITC is 490 nm and that for PE is 488 nm and 514 nm. FITC and PE were the two fluorescent probes used in this study.

When laser light hits each cell in the stream of cells passing the observation point, light is scattered and fluorescent markers are excited. Scattered light and light from the fluorescence is collected, detected, and measured. Although measurement of fluorescent pulses is of primary interest in this study, a short discussion on the uses of measuring scattered light is useful.

D - 2.2 Scattered Light

The presence of a cell in the path of the laser beam causes the light to scatter. Light scatter is classified as forward (2° to 18° relative to the laser path) or right angles (90°) scatter. Light scatter in the forward direction, due primarily to diffraction, gives information about cell size. Side scatter, due primarily to refraction, gives information about internal cellular structure, that is, granularity, membrane folding, and the surface form of cells (4).

D - 2.3 Fluorescence

A fluorochrome is a fluorescent compound. When the laser beam, with a wavelength of 488 nm hits the fluorochromes FITC and PE (with their wavelengths of 490 and 488 nm respectively), the flurorochromes absorb energy from the laser beam. The extra energy causes the electrons of the flurorochrome to raise to a higher energy level. Within a short time, the electrons return back to their initial energy level, emitting photons. Photon emission is called fluorescence. When these photons are emitted from the fluorochrome, some energy is lost as heat, thus the fluorescence, after it has emitted photons, contains less energy than the fluorochrome had in the first place. Less energy translates into higher wavelengths. The emission wavelengths for FITC and PE are 520 nm and 575 nm respectively. Because the excitation wavelengths of these two fluorochromes are very close to one another, they can both be excited by the same beam of light. Since their emission wavelengths differ, detection and measurement systems in a flow cytometer can detect the difference between FITC and PE. If FITC and PE are bound to different types of cells, then these different cells can be differentiated from each other. Dual fluorescence refers to the practice of using two fluorescent probes, such as FITC and PE.

There are actually numerous other measurements that can be derived from flow cytometers, for example, light absorption, but these will not be discussed here.

D - 2.4 Collection of Light

The scattered light and emitted fluorescent light (photons) must be directed to the photodetectors. The key is getting as much of this light to the photodetectors as possible without too much additional background light getting detected as well. Optical filters provide the means to collect light while allowing for discrimination between scattered, flurorescent, and background light.

D - 2.5 Light Detectors

Photodiodes are often used to detect forward scatter. Photomultiplier tubes (PMTs) are used to measure right angle scatter and fluorescence. Both photodiodes and PMTs convert light into electric energy.

D - 2.6 Computers

Computers play important roles in processing the signals from light detectors. Flow cytometry is a method that can perform fluorescent analysis on single cells at rates of up to 50,000 cells per minute (5), so the ability of a computer to analyze, very quickly, the information that it is receiving is no small task.

D - 3 Immunophenotyping

In this study, leukocyte counts and lymphocyte subset counts were determined using monoclonal antibody pairs that were ① specific for the cell surface markers that defined their cell lineage, and ② which were bound to either the fluorochrome FITC or PE. See Table D - 1 for a list of the lymphocyte subsets measured and their identifying cell surface marker names, along with the fluorochrome used.

Table D - 1. Cell surface marker and fluorochrome used to identify lymphocyte subset type.

Lymphocyte subset	Cell surface marker and fluorochrome
Total T cells and Total B cells	CD3-FITC/CD19-PE
T_{helper} cells	CD3-FITC/CD4-PE
$T_{cytolytic}$ cells	CD3-FITC/CD8-PE
Leukocytes and Monocytes	CD45-FITC/CD14-PE
Natural killer cells	CD3-FITC/CD16, 56-PE

D - 4 References

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Appendix E

Alternative hypotheses

This appendix will attempt to highlight an hypothesis that may help explain the apparent connection between endurance training and immunity. Since the present study was performed, some recent research has been published. One idea in particular, that of exercise induced glucocorticoid inhibition of cytokine transcription, will be discussed here.

E - 1 How do Glucocorticoids Exert their Immunomodulatory Actions?

Glucocorticoids, such as cortisol, are steroid hormones. They mediate their effects through the classic model of steroid-hormone control (1). Glucocorticoids arrive at their target cell via the circulatory system. Being lipid soluble, they diffuse across the lipid bilayer membrane of the cell, and interact with their receptor protein on the nuclear membrane. Activation of the hormone-receptor complex enables the receptor to alter RNA transcription of targeted proteins by increasing the affinity of the receptor protein to bind to DNA (2). Glucocorticoids are believed to alter transcription by interacting with enhancer or regulatory glucocorticoid response elements upstream or downstream from the promoter regions (2). This regulates the transcriptional efficiency of specific promoters (3). Another proposed mechanism for the suppressive actions of glucocorticoids on transcription rates is that glucocorticoid-bound receptors bind to DNA sequences, overlapping binding sites essential in transcription, thus preventing their function (3). Although the classic model of steroid-hormone control applies for glucocorticoid immunosuppression, the actual DNA sites at which glucocorticoids act are not well known.

Two means of glucocorticoid immunosuppressive control are: ① interference with an immune cell's ability to turn on genes required to mount an effective immune response (4, 5), and ② induction of thymocyte and peripheral T cell programmed cell death (apoptosis).

E - 2 Inhibition of an Effective Immune Response.

Cytokines and cell adhesion molecules play primary roles in the initiation and maintenance of an immune response. In order for these molecules to be formed, transcriptional activation of their genes is necessary. Glucocorticoids have been shown to reduce circulating populations of leukocytes, resulting in monocytopenia and lymphocytopenia (6). This interference with the accumulation of leukocytes at an inflammatory site has been proposed to be partly due to glucocorticoid inhibition of interleukin-2 (IL-2) production (7). In addition to IL-2, glucocorticoids have been reported to inhibit transcription of the cytokines gamma-interferon (IFN-γ) (8), interleukin-1 (IL-1) (9, 10), tumour necrosis factor alpha (TNFα) (^{11}), and interleukin-6 (IL-6) (12). Auphan et al. (4) suggested that glucocorticoids inhibit synthesis of almost all known cytokines and several cell surface molecules required for immune function. Glucocorticoids have been shown to inhibit immune function orchestrated by cytokines. An example is the shifting of the T cell response from inflammatory T_H1 type to the helper T_H2 (see a description of these in Appendix C) type, thus inhibiting numerous inflammatory processes, including: antigen presentation, lymphocyte, monocyte, and granulocyte chemotaxis, and extravasation and migration of inflammatory cells (8). Since cytokines act as mediators of immune responses, interference in their production has been promoted as one of the possible routes through which glucocorticoids exert their immunosuppressive function.

An explanation of the pathway linking glucocorticoids and suppression of gene transcription of molecules relevant in the immune response follows. Transcription factors are proteins that bind to gene enhancers, thereby directly or indirectly affecting the initiation of transcription (13). Two transcriptional factors affected by glucocorticoids are activator protein 1 (AP-1) and nuclear factor kappa B (NF-κB). AP-1 is involved in lymphokine gene induction (14). NF-κB is involved in the induction of several immunoregulatory genes, including those encoding IL-1, IL-2, IL-3, IL-6, IL-8, TNFα, IFNγ, granulocyte-macrophage colony-stimulating factor, class I and class II MHC complexes, endothelial leukocyte adhesion molecule (ELAM-1), and intercellular adhesion molecule 1 (IAM-1) (15). Glucocorticoids inhibit both AP-1 (16) and NF-κB activation (4, 5), thus transcription of the molecules that these factors code for is suppressed. Glucocorticoid inhibition of NF-κB is mediated by the induction of the inhibitor kappa B alpha (IκBα) inhibitory protein, which traps activated NF-κB in inactive cytoplasmic complexes. This decrease in NF-κB is predicted to markedly decrease cytokine secretion and thus effectively block the activation of the immune system (4, 5).

E - 3 Apoptosis

A second mechanism of glucocorticoid immunosuppression is that of glucocorticoid induced apoptosis (8, 17). Tuosto et al. (18) reported that dexamethasone, a synthetic glucocorticoid, causes apoptosis in mature human T cells. Mature resting T cells are approximately 100 times less sensitive to glucocorticoid induced apoptosis than immature thymocytes or antigen-activated T cells (19). The mechanism by which apoptosis is activated by glucocorticoids is not well known (20).

E - 4 Conclusion

It is apparent from understanding these two means of glucocorticoid induced immunomodulation, that an immunologically challenged organism has a greater chance of being affected by glucocorticoid immunosuppression than does a non-immunologically challenged organism. The reasons are that inhibition of the transcription factors, AP-1 and NF-κB, may play an important role in suppressing an immune response to an antigenic challenge. Also, antigen activated or immature thymocytes are 100 times more sensitive to glucocorticoid induced apoptosis than mature resting T lymphocytes (19). It has been reported that athletes are particularly vulnerable to virus and/or bacterial infections during and immediately following a stressful exercise session or competition (21, 22), and glucocorticoids are produced during and following an acute exercise bout (23). Animal studies have demonstrated that fatiguing exercise at the time of infection increased an animal's susceptibility (24, 25). It is possible that exercise-induced elevated glucocorticoids could suppress an immune response to an antigenic challenge.

When this present study was conducted, it was known that glucocorticoids had potent effects on immune system cells and organs (26). Glucocorticoid receptors had been found on immune tissue and cells, but the precise mechanism of action was still unclear. Apoptosis was a popular proposed means for glucocorticoid induced immunosuppression, but research by Auphan et al. (4) and Scheinman et al. (5) published in the year following the completion of this study presented logical mechansims. The choice to measure resting counts of circulating lymphocyte subsets and cytokines was made with the assumption that immunosuppression would be reflected as depressed numbers of these cells and molecules. In retrospect, with the added knowledge about glucocorticoid-induced immunosuppression, measuring an athlete's strength of immune response to an imposed antigen in addition to measuring resting counts of lymphocyte cells and cytokines may have offered more information. Unfortunately, the technique to measure an athlete's strength of immune response to an imposed antigen has not been developed. This question of how to measure the strength of an immune response, along with the fact that imposing an antigenic challenge on humans is questionable ethically, are two issues that need to addressed if this route of research is to be pursued.

E - 5 References

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